The Mechanism of Action and Therapeutic Potential of S-Nitrosothiols as Novel Nitric Oxide Donor Drugs

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Figure: scanning electron micrograph of blood cells adhering to the intimal surface of a rabbit carotid artery, that has been denuded of vascular endothelium by balloon angioplasty. White bar represents 10 micrometres

Declaration

I hereby declare that the work described in this thesis was performed entirely by myself, and that it has not been accepted in any previous application for a degree.

Mark R. Miller

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There are one or two people I would like to thank. At the risk of sounding like Gwyneth Paltrow at an Oscars ceremony, I wish to do this with a modicum of detail so that I don't skim over anyone important. Bear with me, I promise there won't be any tears!

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Right then, who's round is it .....?

Extracts from early case studies featuring the use and limitations of the NO donors, amyl nitrite and glyceryl trinitrate, in the treatment of angina pectoris

'Few things are more distressing to a physician than to stand beside a suffering patient who is anxiously looking to him for that relief from pain which he feels himself utterly unable to afford...

'Perhaps there is no class of cases in which such occurrences as this take place so frequently as in some kinds of cardiac diseases, in which angina pectoris forms at once the most prominent and the most painful and distressing symptom...

'Brandy, ether, chloroform, ammonia, and other stimulants have hitherto been chiefly relied upon for the relief of angina pectoris; but the alleviation which they produce is but slight...

'On pouring ten drops of the [amyl] nitrite on a cloth and giving it to a patient to inhale...the pain completely disappeared, and generally did not reappear till its wonted time next night.'

Dr T.L. Brunton, The Lancet, 1867.

'About three minutes after the dose [of glyceryl trinitrate] had been placed on his tongue he noticed a sensation of fulness in both sides of the neck, succeeded by nausea. For a moment or two there was a little mental confusion, accompanied by a loud rushing noise in the ears, like steam passing out of a kettle...

'...there was for a few minutes unconsciousness accompanied by convulsive action of the muscles of the face, and stertorous breathing. After swallowing some brandy-and-water, she vomited, and the unpleasant symptoms gradually subsided...

'One afternoon whilst seeing outpatients, I remembered that I had the bottle in my pocket. Wishing to taste it, I applied the moistened cork to my tongue...The pulsation rapidly increased and soon became so severe that each beat of the heart seemed to shake my whole body...

'Sometimes it produced curious fits of gaping; she went on yawning and yawning, and seemed as if she would never stop...

'A friend, who for some days took four drops every three or four hours, informs me that at times it affected his head "most strangely"...

'She became quite insensible, and once remained so for ten minutes. Each fainting-fit was "followed by cold shivers," which "shook her violently all over." Her husband and friends were greatly alarmed, but she thought on the whole it had done her good...

'...he called attention to the fact that the administration of the drug [glyceryl trinitrate] always caused an increased flow of urine. On examination...the urine was seen spouting from the extremity of each ureter in a little jet some three or four inches high. Ordinarily the urine dribbles away drop by drop and never spouts out. The patient was much amazed, and said that in the whole course of his life he had never known it go on in that way. If he took beer or spirits it would increase the flow, but this, to use his own expression, "licked everything."'

Dr W. Murrell, The Lancet, 1879.

Abstract

Nitric oxide (NO) is a powerful vasodilator that is synthesised by the endothelial cells that line healthy blood vessels. NO is now recognised to have many roles in the cardiovascular system, regulating blood vessel tone and the activity of platelets and inflammatory cells. Reduced bioavailability of endothelium-derived NO is a feature of many cardiovascular diseases and the delivery of exogenous NO is an attractive therapeutic option. Conventional NO donors such as the organic nitrates have been used for many years, but they have limitations that restrict their clinical use. S-Nitrosothiols are NO donor drugs that are emerging as potential therapeutic alternatives to conventional NO donors, although their mechanism of action is poorly understood. Recently, two novel S-nitrosothiols, N-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-ß-D-glucopyranose (RIG200) and S-nitroso-*N*-valerylpenicillamine (SNVP), have been described to have selectivity for endothelium-denuded blood vessels. Therefore, they are particularly appealing in the treatment of conditions where the vascular endothelium is damaged. This thesis describes experiments which further elucidate the mechanism of action and therapeutic potential of these novel S-nitrosothiols, by comparison to conventional NO donors.

NO donors were administered specifically to the lumen of isolated rat femoral arteries in an *in vitro* perfusion system. Pretreatment with inhibitors of the NO:soluble guanylate cyclase (sGC) pathway were used clarify the mechanism of action of NO donors. The induction of tolerance was investigated by continuous perfusion of NO donors for 20 h. The therapeutic potential of SNVP was investigated in an *in vivo* rabbit model of balloon angioplasty, by measuring the adhesion of radiolabelled platelets to vessels following endothelial damage

The key findings were that S-nitrosothiol-mediated vasodilator activity is a complex process that is dependent on intracellular antioxidant molecules, particularly glutathione. The site of NO release influenced both its susceptibility to inactivation by

reactive oxygen species and the extent of sGC involvement. Evidence is also presented to show that the novel S-nitrosothiols do not induce self-tolerance with prolonged administration and remain fully active in nitrate-tolerant vessels.

Experiments then focused on the sustained actions of lipophilic S-nitrosothiols in models of endothelial damage. Firstly, glyco-SNAP, an analogue of RIG200 with poor lipophilicity, failed to produce a sustained vasodilatation in endothelium-denuded rat femoral arteries. This result lends weight to the hypothesis that lipophilic S-nitrosothiols exert a sustained vasodilatation in arteries with damaged endothelium through retention in lipid-rich sub-endothelial layers. In the model of balloon angioplasty, the conventional NO donor, glyceryl trinitrate, had no significant effect on platelet adhesion to damaged carotid arteries and also caused a substantial and potentially undesirable fall in blood pressure. In contrast, SNVP caused a >60% reduction in platelet adhesion at a concentration that had minimal effects on blood pressure.

S-nitrosothiols represent an attractive alternative to conventional NO donor drugs. In particular, lipophilic S-nitrosothiols such as RIG200 and SNVP have advantages over nitrates, as they do not engender vascular tolerance and are selective for vessels with a damaged endothelium. SNVP prevents platelet adhesion to vessels that have undergone angioplasty and subsequently may reduce restenosis in the long-term. The results suggest that these novel compounds may have applications in the treatment of a range of cardiovascular conditions, including atherosclerosis, thrombosis and the prevention of restenosis following bypass grafting and stenting.

Publications and Presentations

Publications

MILLER, M.R., ROSEBERRY, M.J., MAZZEI, F.A., BUTLER, A.R., WEBB, D.J. & MEGSON, I.L. (2000). Novel S-nitrosothiols do not engender vascular tolerance and remain effective in glyceryltrinitrate-tolerant rat femoral arteries. *European Journal of Pharmacology*, **408**, 335-343.

MILLER, M.R., ROSEBERRY, M.J., WEBB, D.J. & MEGSON, I.L. (2002). Extracellular NO release mediates the soluble guanylate cyclase-independent vasodilator action of spermine NONOate: Comparison with other NO donors in isolated rat femoral arteries. *British Journal of Pharmacology*, **submitted for publication.**

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List of Abbreviations

AC	adenylate cyclase
ACE	angiotensin converting enzyme
ACh	acetylcholine
ADP	adenosine 5'-diphosphate
ADMA	asymmetrical N^G,N^G-dimethyl-L-arginine
AE1	anion exchange protein 1
ANOVA	analysis of variance
atm	atmospheres of pressure
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
BP	blood pressure
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CABG	coronary artery bypass grafting
cAMP	cyclic adenosine-3',5'-monophosphate
cGMP	cyclic guanosine-3',5'-monophosphate
CHF	chronic heart failure
csPDI	cell surface protein disulphide isomerase
Cys	cysteine
DEA/NO	2-(N,N-diethylamino)-diazenolate-2-oxide
DETCA	diethyldithiocarbamic acid
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
dpm	decays per minute

DQ	duroquinone
EA	ethacrynic acid
EDRF	endothelium-derived relaxing factor
EM	electron microscopy
eNOS	endothelial nitric oxide synthase
glyco-SNAP	N-(S-nitroso-N-acetylpenicillamine)-2-amino-2-deoxy-α,β-D-glucopyranose
GP	glycoprotein
GSH	glutathione
GSL	Griffonia Simplicifolia Lectin
GSNO	S-nitrosoglutathione
GST	glutathione-S-transferase
GTN	glyceryltrinitrate
GTP	guanosine-5'-triphosphate
Hb	haemoglobin
HPLC	high performance liquid chromatography
HQ	hydroquinone
HR	heart rate
Hep-Sal	heparinised-saline
iNOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate
ISDN	isosorbide dinitrate
ISP	isoprenaline
k/s	krebs or saline
L-arg	L-arginine
L-NMMA	N^G-monomethyl-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester

MB	methylene blue
MERC	2-mercaptopyridine
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NANC	non-adrenergic, non-cholinergic
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO^-	nitroxyl ion
NO^+	nitrosium ion
NO_2	nitrogen dioxide
NO_2^-	nitrite
NO_3^-	nitrate
N_2O_3	dinitrogen trioxide
N_2O_4	dinitrogen tetroxide
NONOate	diazeniumdiolate
NOS	nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
O_2^-	superoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one
$ONOO^-$	peroxynitrite
PBS	phosphate buffer solution
PDE	phosphodiesterase
PE	phenylephrine
pGC	particulate guanylate cyclase
PGI_2	prostacyclin
PKG	cGMP-dependent protein kinase
PRP	platelet-rich plasma
PTCA	percutaneous transluminal coronary angioplasty

RIG200	N-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-ß-D-glucopyranose
RSH	thiol
RSNO	S-nitrosothiol
RSSR	disulphide
SCB	sodium cacodylate buffer
sGC	soluble guanylate cyclase
SH	sulphydryl
SIN1	3-morpholinosydnonomine
SNAP	S-nitroso-*N*-acetylpenicillamine
SNO-Alb	S-nitrosoalbumin
SNOC	S-nitrosocysteine
SNO-Hb	S-nitrosohaemoglobin
SNP	sodium nitroprusside
SNVP	S-nitroso-*N*-valerylpenicillamine
SOD	superoxide dismutase
SPER/NO	(Z)-1-{N-[3-Aminopropyl]-N-[4-(3-aminopropyl-ammonio)butyl]-amino}-diazen-1-ium-1,2-diolate
TESPA	3-aminopropyltriethoxy-saline
t-PA	tissue-type plasminogen activator
VSMC	vascular smooth muscle cell
vWF	von Willebrand Factor

Chapter 1

General Introduction

1. GENERAL INTRODUCTION

1.1 INTRODUCTION

The vascular endothelium is a monolayer of cells lining the inside of blood vessels. For many years, it was believed that the endothelium merely acted as a structural barrier, controlling the access of molecules to underlying smooth muscle cells. However, in the last 30 years it has been shown that the endothelium plays a crucial role in the regulation of vascular haemostasis, through the synthesis and release of vasoactive substances, including prostacyclin, thromboxanes, endothelins and growth factors to name but a few. One sustance in particular has attracted a great deal of attention. A powerful vasodilating substance named endothelium-derived relaxing factor (EDRF) was released from endothelial cells on stimulation. In was only in the 1980s that EDRF was identified as the free radical nitric oxide (NO). Subsequently, this 'simple' molecule has been shown to have a staggering array of actions, not just in the cardiovascular system, but in almost every region of the body.

As our understanding of NO developed, it became apparent that NO-related endothelium-dependent relaxations are attenuated in a number of cardiovascular disease states, notably hypertension and atherosclerosis. Therefore, the delivery of exogenous NO to areas of diminished NO activity is an attractive therapeutic option in the management of these common conditions. However, the clinical use of NO donors is limited. Only the organic nitrates and sodium nitroprusside have a clinical use in the management of specific cardiovascular conditions and both have limitations, preventing a wider application. However, an extensive range of NO donors has now been described, each with their own mechanism of NO release to potentially fulfil a range of therapeutic applications. The subject of this thesis is a class of NO donors

called the S-nitrosothiols. In particular, it focuses on several novel S-nitrosothiols which have been shown to have selective actions in endothelial-denuded arteries. If NO can be targeted specifically to sites of endothelial injury, then these compounds will have unique benefits over other NO donors, minimising side effects through targeted delivery of NO to where it is most needed.

1.2 STRUCTURE AND FUNCTION OF BLOOD VESSELS

1.2.1 Structure of the vessel wall

The vessel wall is divided into three layers; the intima, the media and the adventitia (Rhodin, 1980; Fig 1.1).

1.2.1.1 Tunica intima

The intima is the innermost layer of the vessel wall surrounding the vessel lumen. A single layer of endothelial cells covers the inner surface forming the vascular endothelium. These are highly specialised cells that carry out a number of functions. Firstly, they act as a barrier preventing blood components from coming into contact with cells deeper in the vascular wall. In addition, they form a non-reactive surface, preventing the activation and adhesion of certain blood cells such as platelets and immune cells. These cells also act as a means of signal transduction through the synthesis and release a number of regulatory factors, such as prostaglandins, thromboxanes, endothelins, growth factors and nitric oxide (NO). These substances play a role in the contractile state of the underlying smooth muscle cells, regulation of cell proliferation and migration, activation of blood cells and thrombogenicity; which overall maintains vascular homeostasis in response to stimuli.

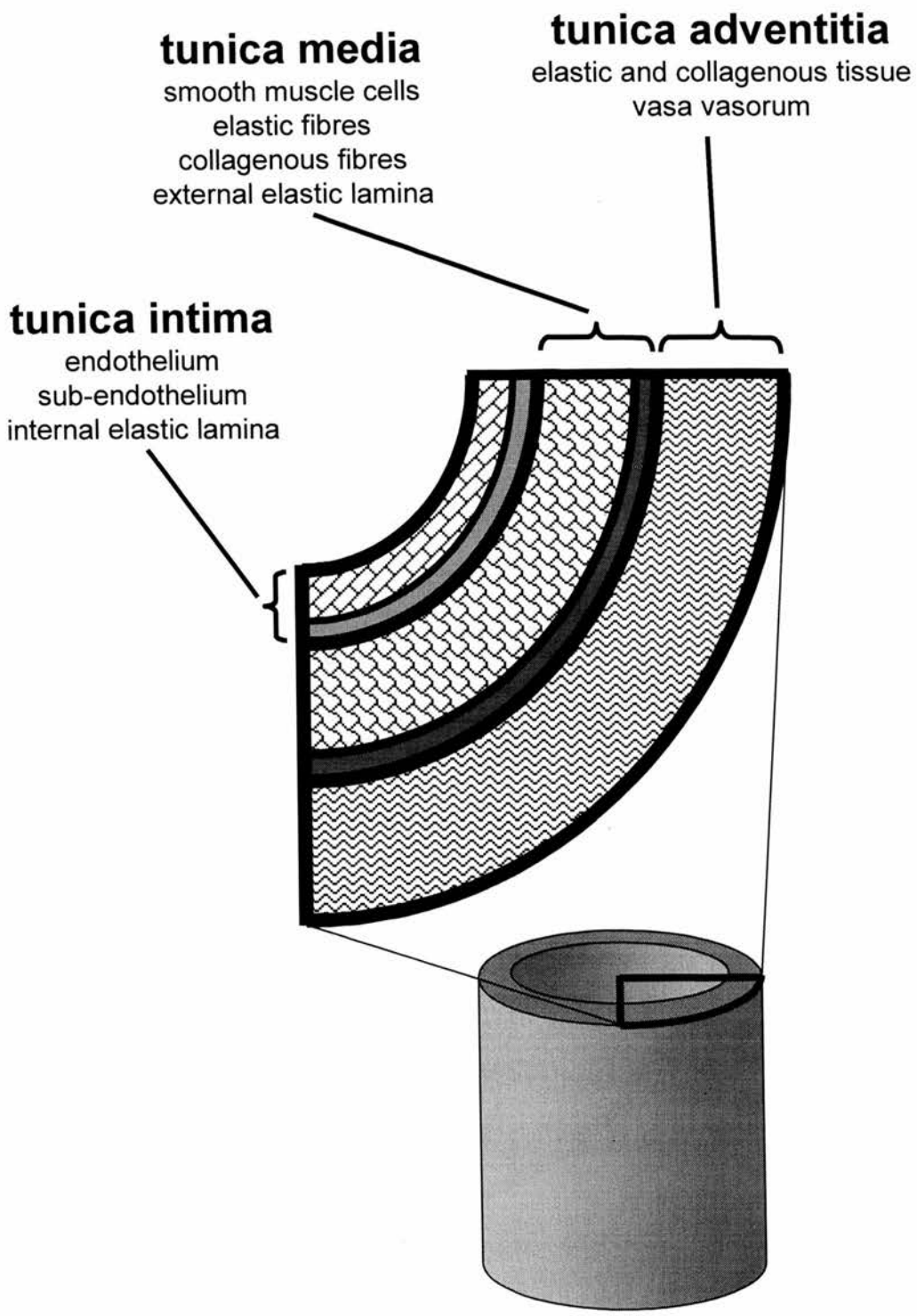


Figure 1.1 Schematic diagram of the structure of blood vessels and the predominate structures within each layer.

The subendothelial layer, between the endothelial cells and the internal elastic lamina, is composed of connective tissue. The size and composition of this region varies with the type of blood vessel, but generally contains bundles of collagen, elastic fibrils and, occasionally, smooth muscle cells and fibroblasts. The internal elastic lamina plays a part in determining the distensability of the vessel wall.

1.2.1.2 Tunica media

The middle layer of blood vessels is composed of layers of vascular smooth muscle cells (VSMCs) between sheets of collagen and elastin fibres. The predominance of VSMCs over elastic layers is determined by the type of blood vessel (i.e. elastic versus muscular arteries). The contractile state of VSMCs is crucial in determining blood distribution and, consequently, is tightly regulated by neural control and an array of humoural factors.

1.2.1.3 Tunica adventitia

The outermost layer forms a sheath over the external elastic lamina that covers the media. The thickness and composition of this layer varies considerably, depending on the vessel type and location. Essentially it consists of fibroelastic connective tissue to provide additional strength and to anchor the vessel in the surrounding tissue. The adventitia of larger arteries houses nerves and minute blood vessels (vasa vasorum) which provide oxygen and nutrients to vessels with thick medial layers.

1.3 THE DISCOVERY OF THE IDENTITY OF EDRF

In 1980, Furchgott & Zawadzki demonstrated that the presence of an intact vascular endothelium was required for acetylcholine (ACh)-mediated vasodilatation of rabbit aortic strips or rings (Furchgott & Zawadzki, 1980). The vasodilator response of acetylcholine could be prevented by removal of the endothelium by gentling rubbing the intimal surface. The authors expanded on these findings by demonstrating that an endothelium-intact strip of aorta stimulated with ACh could induce a vasodilator response in an adjacent denuded strip, suggesting that a factor released from endothelial cells passes to VSMC where it causes vasodilatation. This factor was shown to be distinct from the established endothelial product, prostacyclin (PGI_2), and other arachidonic acid products (see Furchgott, 1984). The factor was named "endothelium-derived relaxing factor" (EDRF). Other groups confirmed the important role of EDRF, using an endothelium-intact tissue as a donor and endothelial-denuded vascular smooth muscle to detect EDRF. The effluent from ACh-perfused aortic segments (Griffith *et al.*, 1984), or endothelial cells culture on microcarrier beads (Cocks *et al.*, 1985; Gryglewski *et al.*, 1986), was shown to relax denuded aortic arteries. These 'simple' bioassay techniques, proved invaluable in the discovery of the chemical identity of EDRF.

In the years following its discovery, the properties of EDRF were rapidly elucidated. Firstly it was established that vasodilatation in response to EDRF was mediated by conversion of guanosine triphosphate (GTP) to cyclic guanosine 3',5'-monophosphate (cGMP) by soluble guanylate cyclase (sGC) in VSMC (Rapoport & Murad, 1983). Soon after the inhibitory effects of the NO scavenger, haemoglobin (Hb), and the sGC inhibitor, methylene blue (MB), demonstrated that EDRF was released basally, as well as by stimulation with agonists such as ACh, bradykinin, adenine nucleotides,

thrombin, substance P, histamine and calcium ionophores (Furchgott, 1984; Martin *et al.*, 1985). Basal release of EDRF was confirmed by monitoring the effect of Hb and MB on vascular tone in unstimulated blood vessels. In addition, release of EDRF could also be increased by mimicking endogenous stimuli such as a pulsatile flow (Rubanyi *et al.*, 1986) and changes in oxygen concentration (Furchgott, 1984).

Bioassay cascade systems were used to estimate the half life of EDRF. Estimates varied considerably, but it was generally concluded that the half life was only a few seconds, confirming the labile nature of EDRF (Griffith *et al.*, 1984; Cocks *et al.*, 1985; Gryglewski *et al.*, 1986) and contributing to the slow progress of its identification. Further experiments also indicated that the short half life of EDRF could be extended by factors which offer protection from superoxide radicals in solution. Superoxide dismutase (SOD), the endogenous enzyme which catalyses the inactivation of superoxide to hydrogen peroxide, prolonged the biological activity of EDRF (Rubanyi *et al.*, 1986), as did cytochrome c, which oxidises superoxide to molecular oxygen. In contrast, superoxide generators, like Fe^{2+} and pyrogallol, reduced the activity of EDRF (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986; Furchgott *et al.*, 1987). Interestingly, it was also shown a few years later that methylene blue inhibits EDRF-mediated vasodilatation partially through the generation of superoxide, rather than inhibition of sGC (Marczin *et al.*, 1992).

Between 1986 and 1988 two groups independently suggested that the free radical nitric oxide (NO) could be EDRF (Ignarro *et al.*, 1986; Furchgott *et al.*, 1987; Ignarro *et al.*, 1988). Despite the similar biological properties of NO, the suggestion was controversial, especially since NO was essentially regarded as a poisonous gas and an environmental pollutant (Denninger & Marletta, 1999). However, conclusive evidence to support this claim came with a chemiluminescence technique was established that could measuring low concentrations of NO. Using ozone to generate a chemiluminescent product (Downes *et al.*, 1976), it was demonstrated that bradykinin-

stimulated endothelial cells generated sufficient chemiluminescence to account for a biologically active amount of NO (Palmer *et al.*, 1987). Direct pharmacological comparison of EDRF and NO demonstrated that both mediate vasodilatation through the generation of cGMP in VSMCs (Rapoport & Murad, 1983) and were susceptible to inhibition by haemoglobin and superoxide generators, but protected by SOD (Palmer *et al.*, 1987; Ignarro *et al.*, 1988).

At present it is generally accepted that EDRF is NO, although some authors have presented data that suggests that EDRF may not be NO *per se*. Initially, the wide range in reported half-lives of EDRF were of some concern, varying between 3 to 50 seconds (Furchgott, 1984; Myers *et al.*, 1990; Moncada *et al.*, 1991), although this may reflect differences in the oxygenation state of the physiological solution used. Also, NO is not anionic and, therefore, does not bind to resins during passage through anion exchange columns. However, EDRF does bind to these columns (Cocks *et al.*, 1985; Long *et al.*, 1987), although it is not clear if this is an artefact caused by a chemical reaction during passage through the column (Moncada *et al.*, 1991). Additionally, NO reacts with haem to produce paramagnetic nitrosyl haem, whereas EDRF does not (Moncada *et al.*, 1991).

One possible explanation for such anomolous findings is that EDRF is an NO-containing compound, or more specifically is *carried* between cells as an NO-adduct. One suggestion is that NO is carried as an iron-sulphur nitrosyl (Vanin, 1991). These complexes are found endogenously and are high capacity NO adducts (Vanin *et al.*, 1997; Ueno & Yoshimura, 2000; Butler & Megson, 2002). The iron content of endothelial cells is reduced during EDRF synthesis and iron-sulphur nitrosyls can be detected after endothelial cell stimulation by electron paramagnetic resonance (Mulsch *et al.*, 1993). However, although the vasodilator effects of these compounds are similar to EDRF in some bioassay systems, the longer duration of the vasodilatation of iron-sulphur nitrosyl contradicts the hypothesis that they are EDRF (Feelisch *et al.*,

1994). Another alternative is that EDRF is carried as an S-nitrosothiol. Thiols are sulphydryl-containing compounds (RSHs) that exist in high concentrations physiologically and their nitrosated form have been identified under physiological conditions (Gaston, 1999). S-nitrosothiols have biological actions similar to EDRF and Myers *et al.* (Myers *et al.*, 1990) suggested that EDRF is the nitrosated form of cysteine (S-nitrosocysteine; SNOC). Certainly, the stability of EDRF in this particular study more accurately represented SNOC than NO, however, the non-specific measurement of NO and NO_2^- may have biased comparisons (Moncada *et al.*, 1991). Other pharmacological studies dispute the suggestion that EDRF is SNOC (Feelisch *et al.*, 1994) and at present it is almost unanimously agreed that EDRF is NO.

1.4 THE NO:sGC PATHWAY

Ever since the discovery of NO as an important physiological regulator, the pathway of its biological action has been the focus of much research. Uncertainty surrounds almost every stage of the pathway , but the generally agreed aspects are summarised in Fig 1.2. Briefly, in blood vessels, stimuli such as shear stress or pharmacological agents acting on receptors, stimulate an increase in cytosolic calcium in endothelial cells. The increase in calcium stimulates the enzyme NO synthase to generate NO, that diffuses, or is carried, out of endothelial cells and into underlying VSMCs. NO binds to, and activates, sGC increasing the generation of cGMP. cGMP stimulates protein kinase G, which ultimately lowers cytosolic calcium in VSMC, leading to the dephosphorylation of myosin light chains. This inhibits contraction, resulting in vasodilatation (Waldman & Murad, 1987; Moncada *et al.*, 1991; Carvajal *et al.*, 2000).

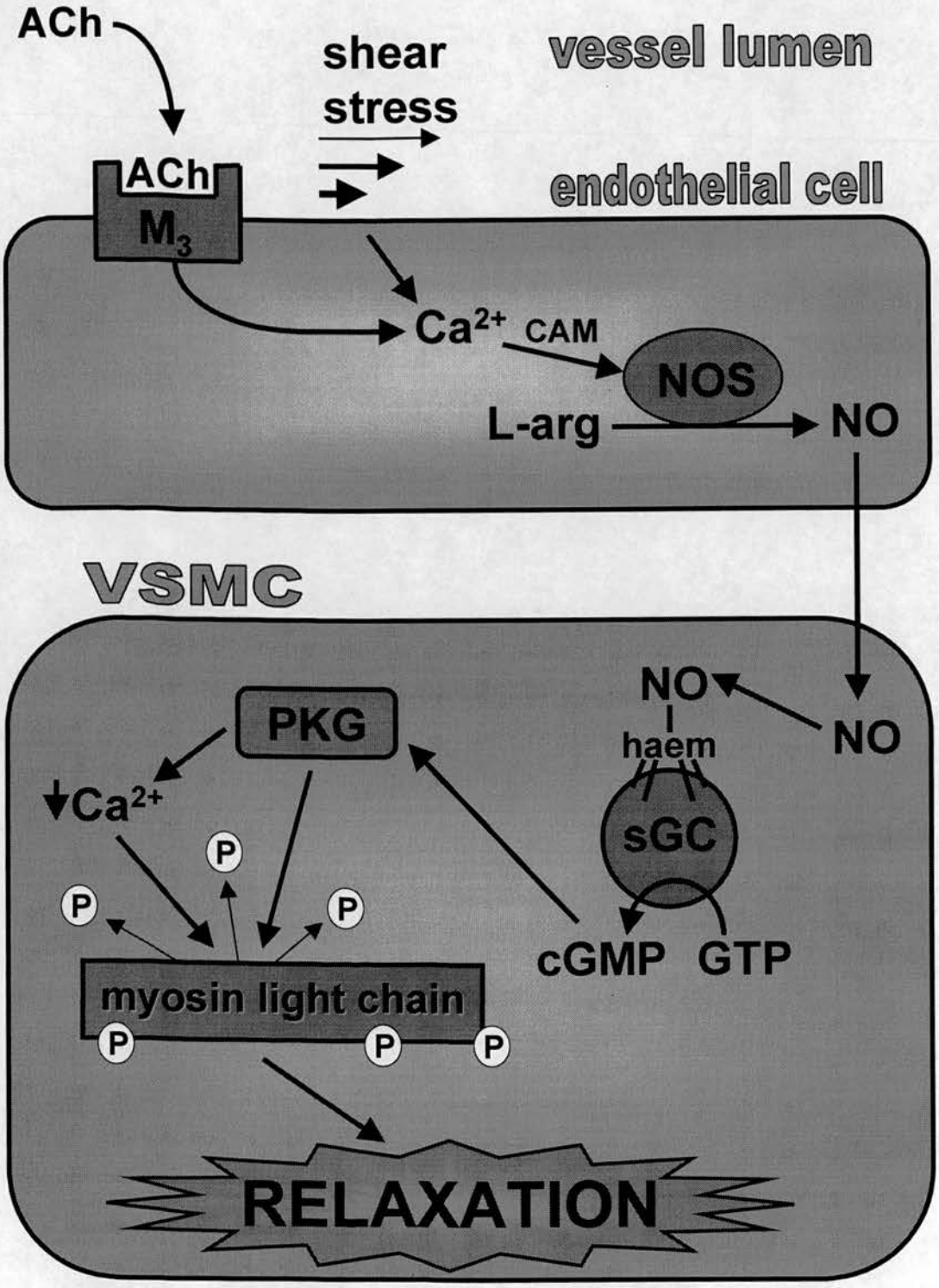


Figure 1.2 Schematic diagram of the NO:sGC pathway in blood vessels.

1.4.1 Induction of NO synthesis

Physiologically, the most important regulator of NO synthesis and release is the shear stress caused by 'drag' of flowing blood across endothelial cell surface (Rubanyi & Vanhoutte, 1986). The mechanism by which the endothelium senses shear stress and translates the signal into NO synthesis has not been fully identified. As stated above, NO synthesis generally requires an increases in endothelial cell cytosolic calcium concentrations. However, NO release has been shown to occur at Ca^{2+} concentrations as low as 10 nM (Mo *et al.*, 1991), suggesting that shear stress may be able to directly stimulate NO production in a calcium-independent manner (Ayajiki *et al.*, 1996; Fisslthaler *et al.*, 2000; Nakano *et al.*, 2000). Ingber (Ingber, 1997) suggest that the force of shear stress itself modulates the cell cytoskeleton via mechanoreceptors, although this theory has not yet been applied to endothelial cells specifically. Additionally, shear stress also influences NO synthesis through slower mechanisms, such as phosphorylation and increased transcription of the NO synthesising enzyme (Vallance & Chan, 2001).

Many endogenous factors carried by the blood, such as histamine, adenine nucleotides, bradykinin, endothelin, serotonin, substance P, thrombin and noradrenaline, can also trigger NO synthesis (Furchgott, 1984; Lerman *et al.*, 1991). All these factors bind to receptors on endothelial cells and ultimately increase cytosolic calcium. A number of exogenous pharmacological agents act in similar ways. Acetylcholine and other muscarinic receptor agonists, as well as calcium ionophores, both produce increases in cytosolic calcium in endothelial cells. Once again, there is evidence of routes of cellular transduction by which agonists can stimulate NO synthesis in a calcium-independent manner (Butt *et al.*, 2000). Regulation is most likely occur by phosphorylation of the NO synthesising enzyme (Butt *et al.*, 2000;

Fisslthaler *et al.*, 2000), or through alteration of enzyme expression and transcription (Nishida *et al.*, 1992; Weiner *et al.*, 1994).

1.4.2 Synthesis of NO

In the endothelium, NO is synthesised enzymatically by NO synthase (NOS; see Sect 1.4.3), using the amino acid L-arginine (L-arg), but not the stereoisomer, D-Arginine, as substrate (Palmer *et al.*, 1988). The terminal guanido nitrogen of L-arg undergoes a five-electron oxidation, followed by cleavage to form free NO and L-citrulline (Stuehr & Griffith, 1992; Fig 1.3). L-citrulline is recycled back to L-arg through a series of enzymatic reactions (Morris & Billiar, 1994), although in the case of endothelial NOS (eNOS) L-arg concentration (150-250 μM) is unlikely to be rate limiting due to the low substrate requirement of NOS (K_m=5-10 μM) (Bult *et al.*, 1999; Hobbs *et al.*, 1999). L-arginine analogues such as asymmetrical N^G,N^G-dimethyl-L-arginine (ADMA), N^G-monomethyl-L-arginine (L-NMMA), and N^G-nitro-L-arginine methyl ester (L-NAME) act as competitive, reversible inhibitors of NOS (Rees *et al.*, 1990; Vallance *et al.*, 1992; Fig 1.4). Use of L-arginine analogues *in vivo*, produces an increase in arterial tone, indicating that NOS synthesises NO basally in the absence of any pharmacological agonist in both animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Gardiner *et al.*, 1990; Chu *et al.*, 1991) and humans (Vallance *et al.*, 1989; Haynes *et al.*, 1993). D-Arginine analogues do not inhibit NOS activity (Palmer *et al.*, 1988).

Oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) are co-substrates (Palmer & Moncada, 1989; Leone *et al.*, 1991), and the reaction also requires a number of co-factors, importantly (6R)-5,6,7,8-tetrahydrobiopterin (BH_4; Rodriguez-Crespo *et al.*, 1996) and Ca^{2+}/calmodulin complex (Busse & Mulsch, 1990; Mayer *et al.*, 1990). N^G-hydroxy-L-guanidine is formed as an intermediate (Stuehr *et al.*, 1991) and analogues of this intermediate may represent potential NO donor drugs (Everett *et al.*, 1998).

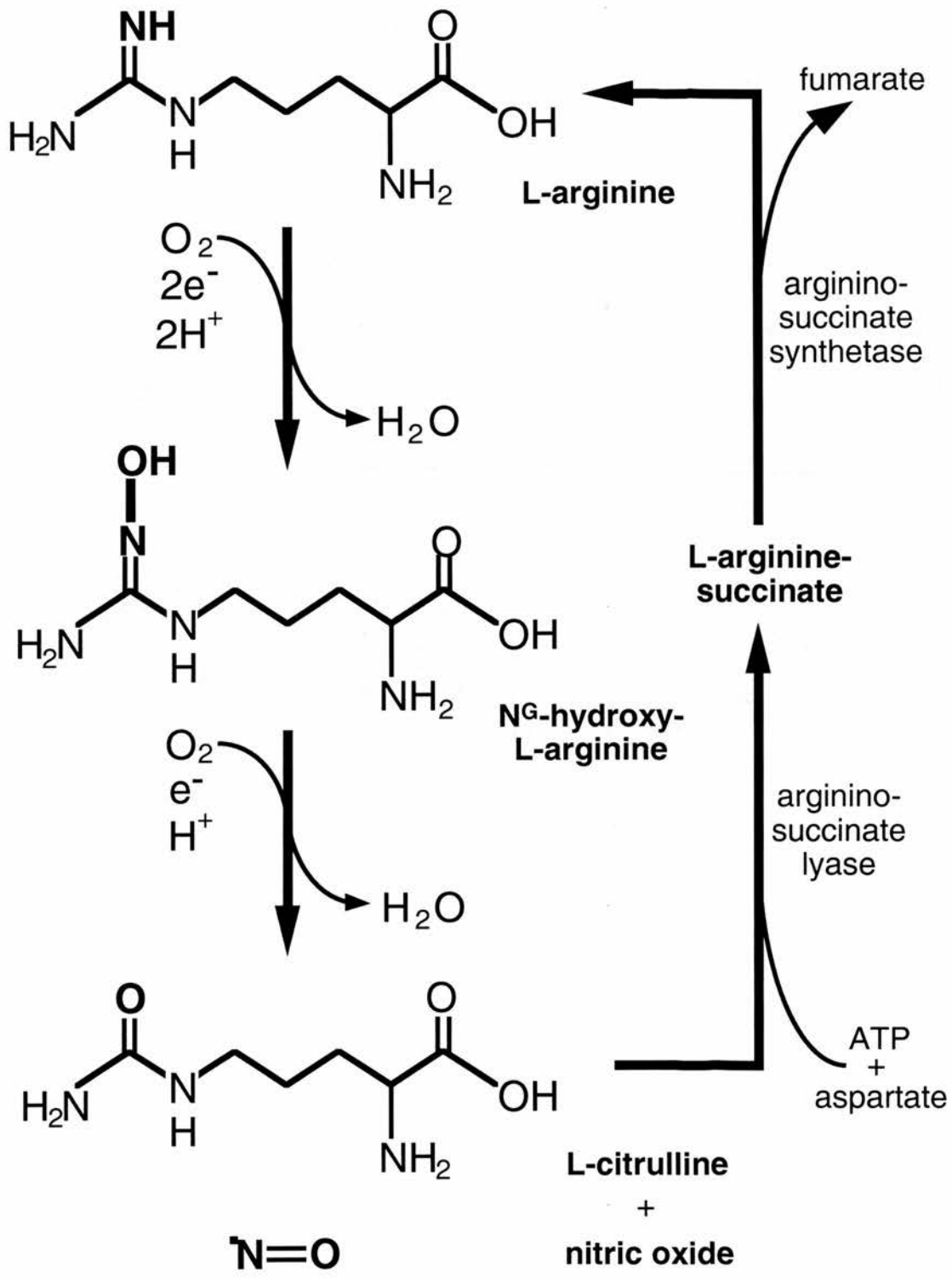


Figure 1.3 Conversion of L-arginine to NO by NO synthase and the recycling of L-citrulline back to L-arginine.

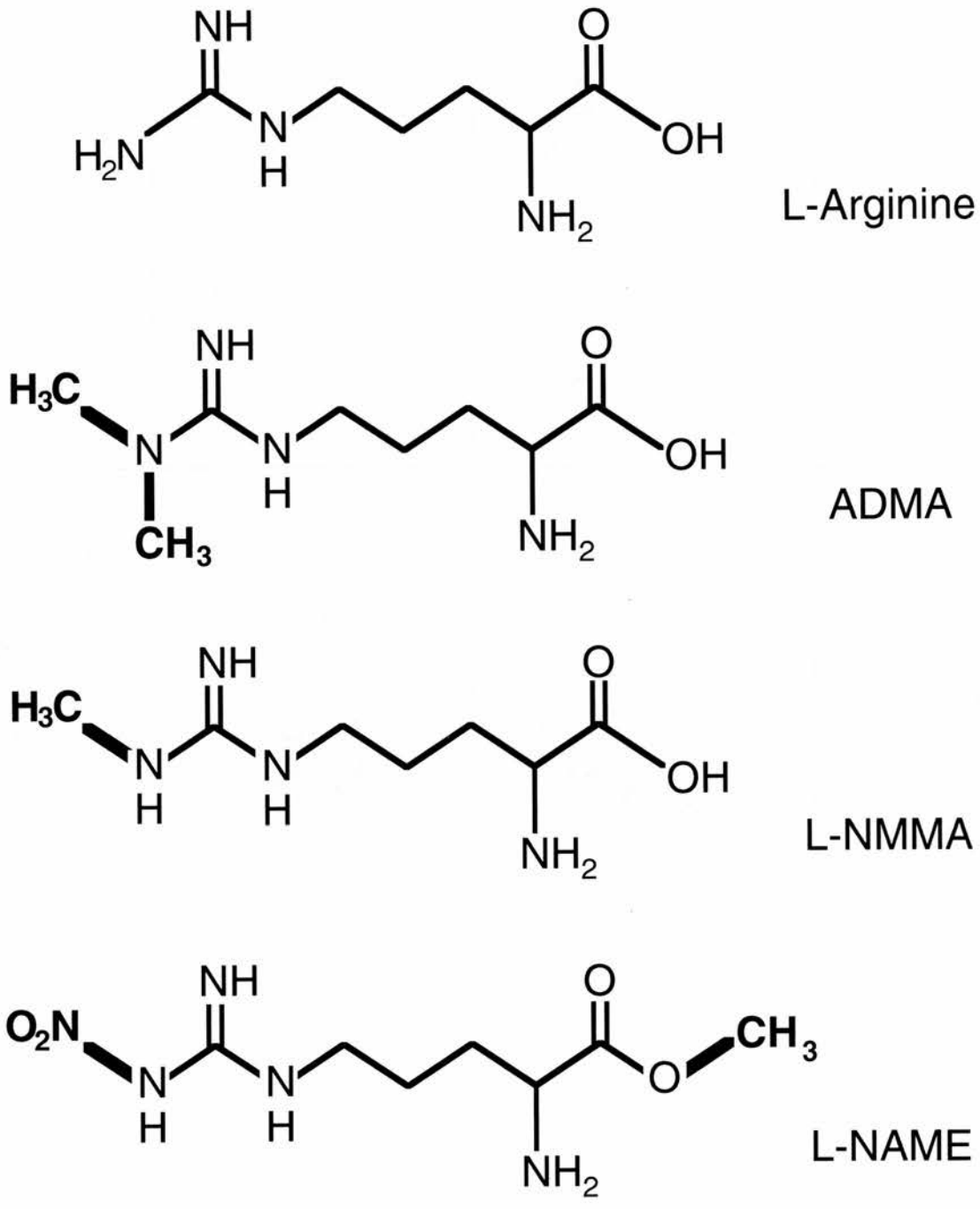


Figure 1.4 Structure of the NOS substrate, L-arginine, and related analogues that inhibit NOS activity.

It has been suggested that NOS actually generates nitroxyl ions (NO^-), the reduced form of NO (Fukuto *et al.*, 1992). NO^- is a poor activator of sGC (Feelisch *et al.*, 1994; Dierks & Burstyn, 1996) and therefore may require prior oxidation to NO by tissue factors (Nelli *et al.*, 2000). The role of NO^- in vasodilatation is controversial and, at present, it is almost universally accepted that NOS generates NO (Stuehr, 1999).

1.4.3 NO synthase

1.4.3.1 NOS isoforms

In mammals, there are three NOS isoforms. Each isoform has its gene on a different chromosome (Nathan & Xie, 1994), although the isoforms share greater than 50% homology (Forstermann *et al.*, 1994).

The first isoform to be purified and cloned was neuronal NOS (nNOS/NOSI) located in the rat and porcine cerebellum (Bredt & Snyder, 1990; Mayer *et al.*, 1990). Since then, nNOS has been found in most areas of the nervous system, as well as in skeletal muscle (Stuehr, 1999). NO generated from nNOS is thought to act as a neurotransmitter and has been shown to exert control on the cardiovascular system, via both the central and peripheral nervous system (Cederqvist *et al.*, 1991; Huang *et al.*, 1995). In addition, nNOS activation may have a role in a number of brain functions, including plasticity and memory (Schuman & Madison, 1991). Endothelial NOS (eNOS/NOS III) is expressed in the endothelium of arteries and veins and was first cloned from bovine aortic endothelial cells (Nishida *et al.*, 1992). The structure and regulation of eNOS are discussed below. Both eNOS and nNOS are found constitutively and are Ca^{2+}/calmodulin-dependent. Inducible NOS (iNOS/NOS II) acts independently of Ca^{2+}/calmodulin and, in general, is synthesised in response to inflammatory stimuli (Stuehr, 1999). It was first purified and cloned from mouse

macrophages (Lowenstein *et al.*, 1992; Xie *et al.*, 1992). Expression can be induced in most cell types in response to cytokines, generating sufficiently high local levels of NO that are cytotoxic. Subsequently, the expression of iNOS in immune cells is thought to act as a defence mechanism, attacking invading pathogens with NO.

1.4.3.2 NOS structure and regulation

All NOS isoforms are composed of two homodimeric subunits, with each monomer containing an N-terminal-oxygenase domain and a C-terminal reductase domain. Dimerization of the two subunits is a pre-requisite for activation and is triggered by the activation of calmodulin with high calcium concentrations. The central region between the domains contains recognition sequences for activated calmodulin (Stuehr, 1999). In the case of eNOS and nNOS, NO synthesis is initiated when the Ca^{2+}/calmodulin complex binds to this central region. Calmodulin is thought to be permanently bound to iNOS, resulting in permanent maximal activation (Cho *et al.*, 1992). Calmodulin is thought to be necessary for electron transfer between the two domains (Bredt *et al.*, 1991; Abu-Soud *et al.*, 1994; Abu-Soud *et al.*, 1994). The reductase domains bind flavins which transfer electrons from NADPH. The electrons are then transferred to the haem iron in the oxygenase domain, via the Ca^{2+}/calmodulin bridge. Following this the two substrates, L-arg and oxygen, bind to the oxygenase domain, catalysing the formation of NO and L-citrulline. The two subunits are thought to align head-to-head, with electrons passing from the reductase domain of one subunit to the oxygenase domain of the other (Fig 1.5).

BH_4 and L-Arg allosterically regulate each other's binding (Klatt *et al.*, 1994). However, a number of other roles have been proposed for BH_4. It has been suggested that BH_4 is necessary for the dimerization of the NOS subunits, through regulation of the redox status of the haem iron (Stuehr, 1999). The actions of BH_4 on the haem

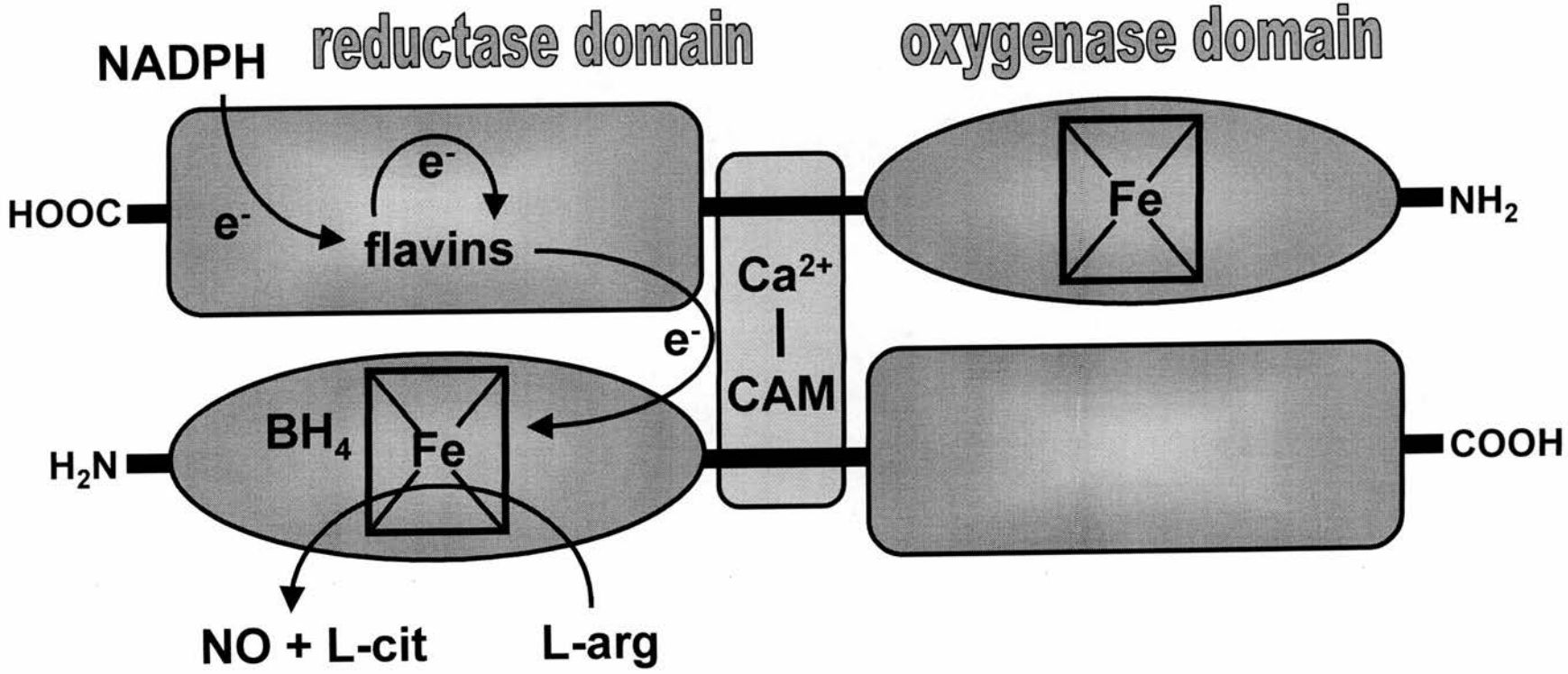


Figure 1.5 Schematic diagram of the NO synthase homodimer. It has been hypothesised that electrons pass from the reductase domain of one subunit to the oxygenase domain of the other subunit, via the Ca^{2+}/calmodulin bridge (Steuhr, 1999)

redox status may instead be necessary for the efficient transfer of electrons to the oxygenase domain (Rodriguez-Crespo *et al.*, 1996; Abu-Soud *et al.*, 1997). In favour of this theory is the finding that in the absence of sufficient BH_4, NOS can generate superoxide rather than NO (Heinzel *et al.*, 1992; Cosentino & Katusic, 1995; Gorren *et al.*, 1996; Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998). This dysfunctional superoxide-generating form of NOS has been linked to a number of cardiovascular conditions (see Sect 1.7.1.2)

L-arginine analogues block NOS activity by different methods (Pou *et al.*, 1992; Abu-Soud *et al.*, 1994). L-NMMA occupies the L-arg binding site, but this coupling does not allow efficient electron transfer to the haem. In contrast, L-NAME prevents the reduction of haem group, making it unreactive to oxygen. The ability of different L-arginine analogues to inhibit NOS varies with the model used and unexpected findings such as L-NMMA acting as a substrate for NOS, or inhibiting L-NAME-induced NOS-inactivation have been reported (Hobbs *et al.*, 1999). The structure of the analogue also affects the relative selectivity for the NOS isoform, albeit somewhat marginally (Stuehr & Griffith, 1992). Attention has focused on inhibitors of NOS which are highly isoform specific, particularly to selectively inhibit iNOS in septic shock or cardiovascular pathophysiology. iNOS-specific inhibitors such as N-(3-(aminomethyl)benzyl)acetamidine (1400W; Garvey *et al.*, 1997; Miller *et al.*, 2000) are currently under experimental investigation (Hobbs *et al.*, 1999). NO itself can also bind to the haem iron, inhibiting NOS activity and acting as a negative feedback (Buga *et al.*, 1993; Abu-Soud *et al.*, 1995; Hurshman & Marletta, 1995).

1.4.4 Soluble guanylate cyclase

1.4.4.1 sGC isolation

The primary target for NO is the cytosolic enzyme sGC, which catalyses the production of the second messenger cGMP. cGMP is the primary mediator of vasodilatation, inhibition of platelet activation and many other actions of NO. Particulate guanylate cyclase (pGC) is found in the membranes of cells. It also catalyses the formation of cGMP, but it is activated by ligands such as atrial and brain natriuretic peptide and not by EDRF/NO (Denninger & Marletta, 1999).

sGC was first identified in mammalian cells in 1969 (Hardman & Sutherland, 1969; Ishikawa *et al.*, 1969; Schultz *et al.*, 1969; White & Aurbach, 1969). It has structural and biochemical similarities to adenylate cyclase (AC). AC catalyses the formation of cyclic adenosine-3',5'-monophosphate (cAMP), mediating many cellular processes, including vasodilatation and inhibition of platelet activation (Denninger & Marletta, 1999). sGC has been studied far less extensively than AC, partly due to the difficulty in isolating and purifying the enzyme (Hobbs, 1997). sGC is highly expressed in the lung and brain, but its isolation from bovine lung is laborious and yields little active protein. Initial purification techniques isolated sGC subunits of variable size depending on species and tissue (Waldman & Murad, 1987). Many early studies using purified sGC, were carried out on haem-free sGC and subsequently showed unusual activity to ligands (Gerzer *et al.*, 1981; Gerzer *et al.*, 1981). The contamination of isolated sGC with detergents, redox compounds and metal chelators during purification could also be a source of erroneous findings. Subsequently, interpretation of results from early studies on purified sGC requires care (Waldman & Murad, 1987). The development of over-expression systems will undoubtedly make the purification of sGC easier (Denninger & Marletta, 1999).

1.4.4.2 sGC structure and regulation

sGC exists as a heterodimer consisting of an α and β subunit. Several isoforms of each subunit have been isolated. Each subunit of sGC can be divided into three sections; the cGMP-catalytic domain, the dimerisation domain and the haem-binding domain (Fig 1.6).

The catalytic domain catalyses the conversion of guanosine-5'-triphosphate to cGMP. Catalysis requires divalent ions, particularly Mg^{2+} and Mn^{2+}. Of these, Mg^{2+} is found in higher concentrations in the cell cytoplasm and probably plays the greater role *in vivo* (Ohlstein *et al.*, 1982). As the name suggests, the dimerisation domain controls the pairing of subunits and probably mediates which isoforms of both subunits can combine (Hobbs, 1997).

The haem-binding domain is the region that is believed to bind NO. Each mole of dimer contains one mole of haem (Wolin *et al.*, 1982), linked to sGC by the four nitrogens of the porphyrin ring and a distal fifth bond to his105 of the β subunit (Wedel *et al.*, 1994; Fig 1.6). NO binds to the iron of haem when it is in the Fe^{2+} state, causing almost a 100 fold increase in basal activation. It is now accepted that haem-deficient sGC is not activated by NO, unless a source of haem is also added (Craven & De Rubertis, 1978; Craven & DeRubertis, 1978; Craven *et al.*, 1979). Oxidation of haem iron to a ferric state prevents NO-induced activation of sGC, whereas reducing agents such as thiols, ascorbate and dithiothreitol, tend to enhance sGC activity (Braughler *et al.*, 1979; Waldman & Murad, 1987). Carbon monoxide also binds to the haem iron of sGC, but causes only a weak activation, and subsequently, tends to act as a competitive inhibitor of sGC (Stone & Marletta, 1995). The exact mechanism of sGC activation by NO is still unclear. It is assumed that NO binds to the haem,

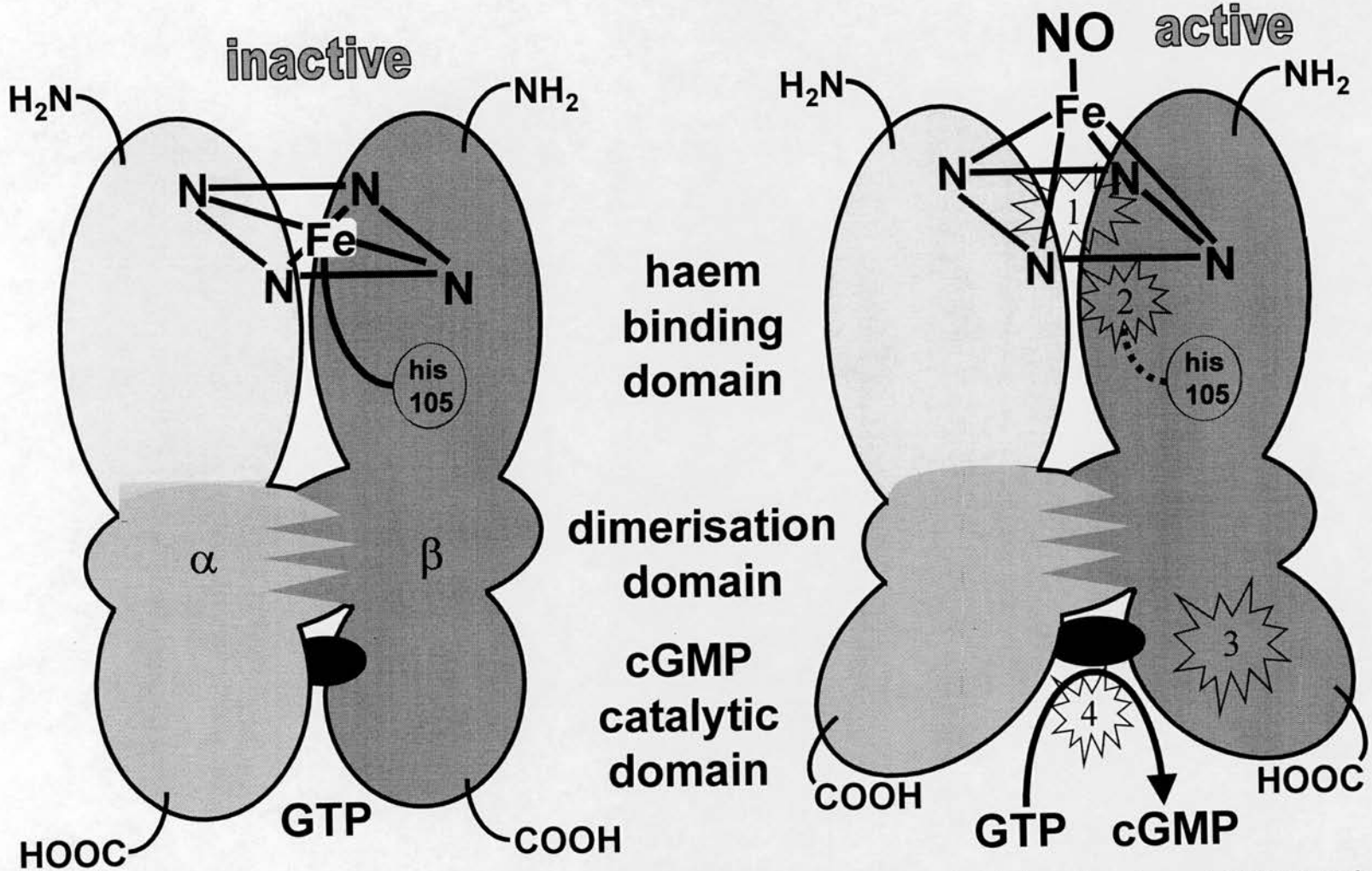


Figure 1.6 Schematic diagram of the structure and activation of sGC. Binding of NO to the haem of sGC, draws haem iron out of the plane of the porphyrin ring (1), breaking the weak bond with his105 (2). Structural changes take place (3) allowing the catalytic domain to generate cGMP (4). Adapted from Hobbs (1997).

drawing iron from the plane of the porphyrin ring. The his105 bond is broken, producing a conformation change in the catalytic domain and stimulating the production of cGMP (Gerzer *et al.*, 1981; Wedel *et al.*, 1994; Lawson *et al.*, 2000).

NO has been shown to be the only nitrogen monoxide capable of activating sGC (Feelisch *et al.*, 1994; Dierks & Burstyn, 1996). NO has a remarkable affinity for sGC and subsequently, it is the deactivation of sGC which remains a mystery. NO-sGC has a half life in the order of minutes (Palmer *et al.*, 1987), which is unusually short for a nitrosyl-haem complex. It has been shown that NO dissociates from sGC within 5 seconds (Kharitonov *et al.*, 1997), but whether this rapid dissociation is an intrinsic factor of sGC following activation, or is mediated by cellular factors, possibly thiols, has not been established (Kharitonov *et al.*, 1997; Brandish *et al.*, 1998).

NO is also believed to bind to additional areas of sGC, although these have not been identified. There are two groups of free thiols in sGC, but at present, there is no evidence that NO reacts with these groups, particularly as NO itself is a poor S-nitrosating agent (Butler *et al.*, 1995). Interestingly, oxidation of these thiols can lead to enhanced activation or cause inactivation of sGC, depending on the group which is oxidised (Braughler, 1983). Thiol alkylating agents (Katsuka *et al.*, 1977; Ignarro & Gruetter, 1980) or exogenous thiols, which form disulphide bridges with the thiols of sGC, inhibit basal and stimulated cGMP production (Kimura *et al.*, 1975; Waldman *et al.*, 1983). These effects can be reversed with dithiothreitol, which restores free thiol groups (Brandwein *et al.*, 1981). Important thiols are juxtaposed in the catalytic site (Kamisaki *et al.*, 1986). Stimulation of the enzyme through haem activation appears to enhance the reactivity of the thiol groups or improve accessibility to thiol modulators, allowing a synergistic activation or promoting deactivation (Kamisaki *et al.*, 1986). sGC also contains a copper ion and it has been shown that exogenous Cu(II) ions and Cu(I) chelators modulate sGC activity (Schrammel *et al.*, 1996). The role of the Cu in

sGC has not been fully explained, but one suggestion is that Cu catalyses the breakdown of S-nitrosothiols to NO, which can then activate sGC (Hobbs, 1997).

It is only in the last 10 years that specific sGC antagonists have been identified. Previously methylene blue had been used as an inhibitor of sGC, but this agent also inhibits NOS and generates superoxide (Marczin *et al.*, 1992; Luo *et al.*, 1995). Therefore, it compounds is of little use in determining any sGC-independent actions of EDRF or NO. Recently, Garthwaite described a novel sGC inhibitor, 1H-[1,2,4,]oxodiazolo[4,3-*a*]quinoxalin-1-one (ODQ; Garthwaite *et al.*, 1995) that has been extremely useful in clarifying the role of sGC. ODQ does not generated superoxide or inhibit NOS, AC or pGC activity. It is generally assumed that ODQ is an irreversible inhibitor of sGC through oxidation of the haem of sGC (Schrammel *et al.*, 1996), although the exact mechanism or nature of inactivation of sGC has yet to be identified. One limitation is that ODQ cannot be used systemically *in vivo*, as ODQ reacts with haemoglobin and binds to myoglobin of the heart (Hobbs, 1997; Wegener *et al.*, 1999).

1.4.5 Targets for cGMP

cGMP has numerous targets in vascular smooth muscle cells. These are discussed below in three main categories (Carvajal *et al.*, 2000; Fig 1.7).

1.4.5.1 Protein kinase G

The main mechanism of cGMP-induced vasodilatation is believed to be via activation of cGMP-dependent protein kinase (PKG), ultimately leading to reduced levels of cytosolic calcium.

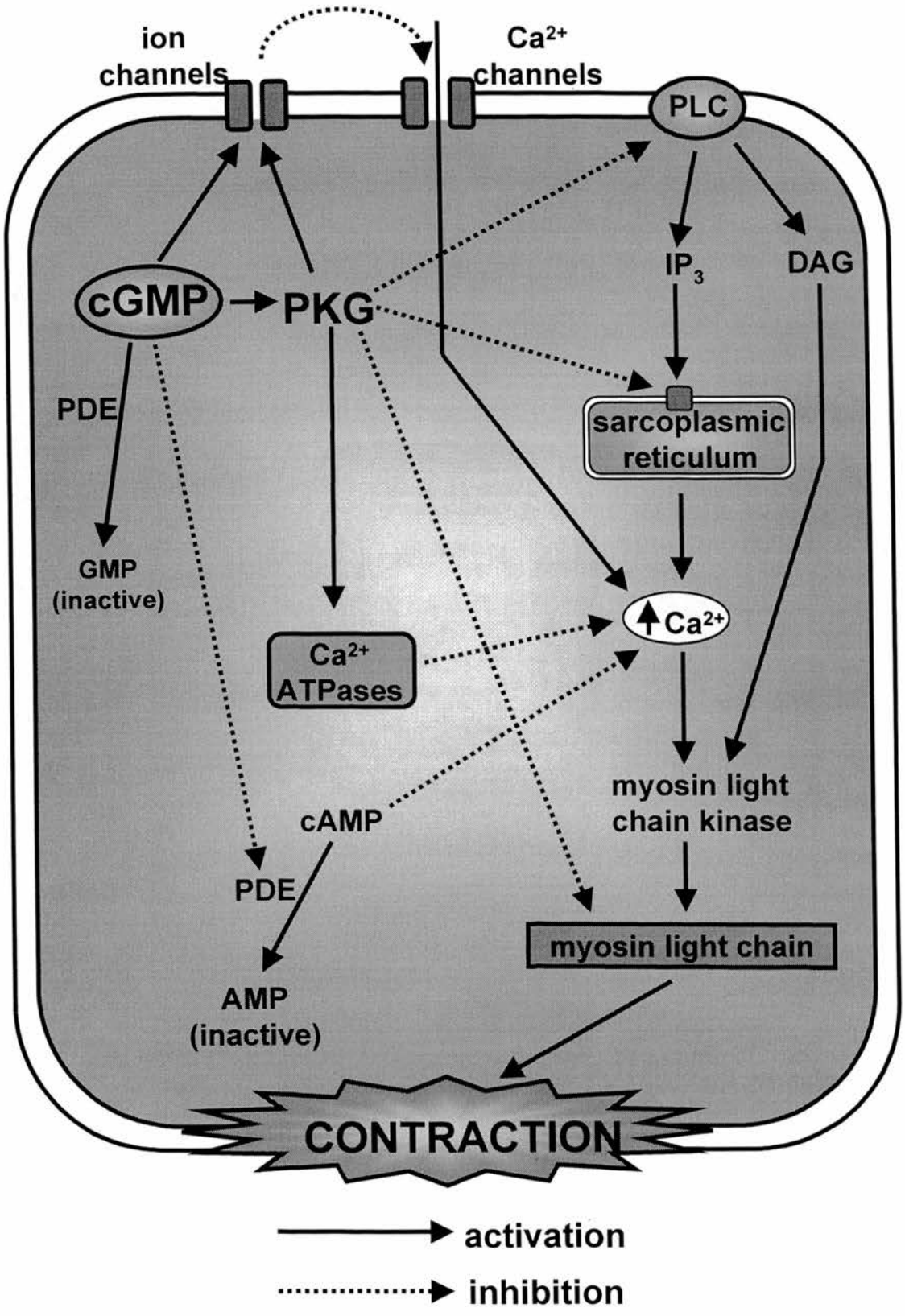


Figure 1.7 Schematic diagram of the mechanisms by which cGMP inhibits the contraction of vascular smooth muscle.

One pathway of Ca^{2+} release is the inositol 1,4,5-triphosphate (IP_3) pathway. IP_3 acts on receptors on the sarcoplasmic reticulum, releasing calcium from sarcoplasmic stores. Calcium binds to calmodulin forming a complex that activates myosin light chain kinase in VSMCs. This kinase phosphorylates myosin light chains, triggering smooth muscle contraction (Walter, 1989).

Like other protein kinases, PKG phosphorylates proteins, regulating their activity. For example PKG inhibits IP_3 production, as well as the co-product, diacylglycerol, another activator of myosin light chain kinase, by phosphorylation of phospholipase C (Rasmussen *et al.*, 1987; Waldmann & Walter, 1989; Lincoln & Cornwell, 1993). PKG also phosphorylates the IP_3 receptor, downregulating its activity (Komalavilas & Lincoln, 1996). Additionally, PKG activates plasmalemmal and sacroplasmic reticulum Ca^{2+}-ATPases, which promote cellular efflux of Ca^{2+} and the uptake of Ca^{2+} into intracellular stores, respectively (Furukawa *et al.*, 1988; Cornwell *et al.*, 1991). PKG also regulates the activity of several plasma membrane ion channels, preventing the influx of Ca^{2+} ions from the extracellular space into the cytosol (Tanaka *et al.*, 1998; Yamakage *et al.*, 1996; Tewari & Simard, 1997). Finally, in some tissues PKG decreases the sensitivity of VSMC contractile apparatus to Ca^{2+} (Chen & Rembold, 1992).

Therefore, PKG acts to 1) downregulate the production of factors releasing Ca^{2+}, 2) downregulate the activity of the receptor for Ca^{2+}-releasing factors, 3) promote uptake of cytosolic Ca^{2+} into intracellular stores, 4) promote efflux of Ca^{2+} out of the cell, 5) decrease the sensitivity of contractile apparatus to cytosolic Ca^{2+} (Lohmann *et al.*, 1997; Carvajal *et al.*, 2000).

1.4.5.2 Regulation of plasma membrane channels

cGMP can regulate ion channels on the plasma membrane independently of PKG, leading to hyperpolarisation. Hyperpolarisation of the cell membrane reduces the open

time of voltage-gated Ca^{2+} channels, resulting in a reduction in cytosolic Ca^{2+}. Some channels are directly cGMP-gated, although this type of channel has yet to be found in VSMCs (Zagotta & Siegelbaum, 1996).

1.4.5.3 Phosphodiesterases

Phosphodiesterases (PDEs) are a large class of enzymes which catalyse the hydrolysis of nucleotides such as cGMP and cAMP. Several isoforms (types II, III & V) hydrolyse cGMP, but the binding of cGMP also influences the activity of the PDE (Carvajal *et al.*, 2000; Degerman *et al.*, 1997). In vascular tissue and platelets, cGMP inhibits activity of PDEs which control the hydrolysis of cAMP, extending the action of cAMP. Therefore, cGMP and cAMP may act synergistically to induce vasodilatation and inhibition of platelet activation. At present, PDEs are of great interest due to the success of the anti-impotence drug, sildenafil (Viagra; Vallance, 1999). Sildenafil inhibits PDE V, enhancing cGMP-mediated vasodilatation and increasing blood flow to the corpus cavernosum - a particularly PDE V rich area of the body (Goldstein *et al.*, 1998; Moreland *et al.*, 1998).

1.4.6 cGMP-independent actions of NO

It has long been known that very high concentrations of NO (e.g. produced by iNOS) have cGMP-independent actions (see Sect 1.6.3), but it has become clear that much lower concentrations of NO may also have cGMP-independent effects. NO and NO donor drugs have been shown to cause vasodilatation (Homer & Wanstall, 2000; Wanstall *et al.*, 2001) and inhibition of platelet aggregation (Gordge *et al.*, 1998; Sogo *et al.*, 2000) which is only partially mediated by cGMP. The identity of these mechanisms has yet to be conclusively identified. However, candidates include voltage-sensitive calcium channels (Travis *et al.*, 2000), calcium dependent potassium

channels (Mistry & Garland, 1998; Plane *et al.*, 1998; Homer & Wanstall, 2000; Plane *et al.*, 2001), sarcoplasmic reticulum Ca^{2+}-ATPases and Na^{+}/K^{+}-ATPases (Gupta *et al.*, 1994; Homer & Wanstall, 2000). In addition, modifiers of protein sulphydryl groups inhibit NO-mediated changes in the activity channels (Bolotina *et al.*, 1994; Campbell *et al.*, 1996; Gordge *et al.*, 1998). This suggest that NO alters channel activity through modification, most likely by S-nitrosation, of these thiol groups. The cGMP-independent actions of NO are discussed in more detail in Chapter 3.

1.5 NO BIOCHEMISTRY

The common assumption that, because of its small size and lack of charge, NO merely diffuses across intracellular and extracellular spaces, and the membranes that separate them (Lancaster, 1994) may be true, yet it overlooks a number of possible reactions. In comparison to other radicals, NO is relatively unreactive (Beckman & Koppenol, 1996; Denninger & Marletta, 1999). Nevertheless, NO *is* still a free radical and the diverse nature and reactivity of the biological environment presents NO with a plethora of possible reactions that can modulate its activity.

1.5.1 Reaction with molecular oxygen

The reaction of NO with molecular oxygen (O_2) is slow (k=6.6 x10^6 $M^{-2}s^{-1}$) and results in the formation of inactive nitrite (NO_2^-) and nitrate (NO_3^-) (Eqns 1,2; Feelisch, 1991), although nitrite formation predominates (Ignarro *et al.*, 1993). Because the initial step is second order with respect to NO (i.e. 2 molecules of NO react with 1 molecule of O_2), the reaction is slow physiologically, due to low (nM) concentrations of NO (Ford *et al.*, 1993; Kharitonov *et al.*, 1995; Keshive *et al.*, 1996). However, the reaction may be significant in the presence of high concentrations of NO (Beckman &

Koppenol, 1996), which can occur due to the induction of iNOS or through pharmacological intervention.

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (2)$$

The formation of nitrogen dioxide (NO_2) is an intermediate step in the inactivation of NO by O_2 (Butler *et al.*, 1995). The species can interact with additional molecules of NO to produce higher nitrogen dioxides (Eqn 3), which are powerful S-nitrosating agents (see below), and can be hydrolysed to nitrite (Eqn 4).

$$NO + NO_2 \rightarrow N_2O_3 \quad (3)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (4)$$

1.5.2 Reaction with superoxide

In vivo, the reaction of NO with the oxygen-derived free radical, superoxide (O_2^-), is arguably more important in the inactivation of NO (Beckman & Koppenol, 1996). Superoxide is generated as a by-product of cellular respiration and metabolism. However, the high concentrations of Mn and Zn SOD isozymes, together with the hydrogen peroxide metabolising enzyme, catalase limit intracellular O_2^- levels (Freedman & Crapo, 1982; Fig 1.8).

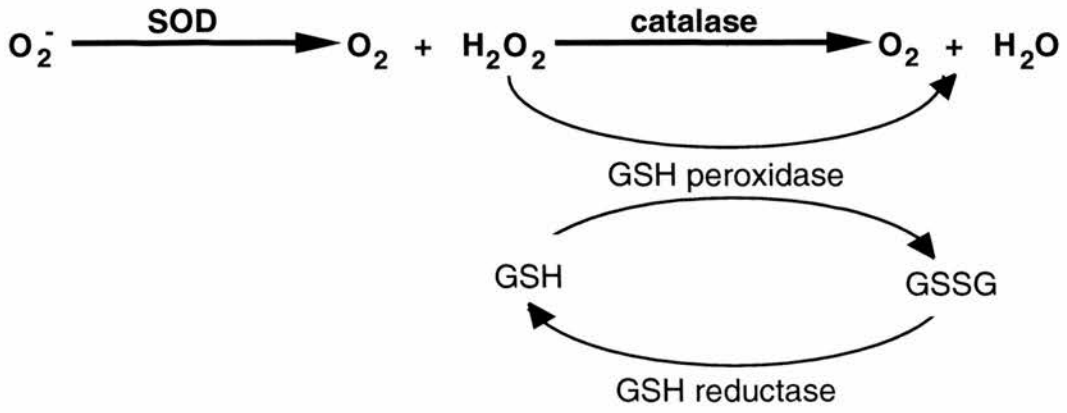


Figure 1.8 Pathway of superoxide deactivation by SOD and catalase.

Antioxidants, such as thiols and glutathione (GSH)-related enzymes, such as GSH peroxidase also contribute to antioxidant defence (Freedman & Crapo, 1982). Superoxide generation is enhanced in a number of cardiovascular conditions, due to enhanced activation of white blood cells, reduced SOD and dysfunctional NOS (see Sect 1.7). Therefore, superoxide levels may become a problem as SOD becomes saturated. Oxygen radicals may also be generated by interaction with free or protein-complexed metal ions such as iron and copper (Freedman & Crapo, 1982; Beckman & Koppenol, 1996). The reaction between NO and superoxide is first order and occurs at essentially a diffusion-limited rate (k=7x10^9 M^{-1}s^{-1}), to form peroxynitrite ($ONOO^-$) (Eqn 5; Huie & Padmaja, 1993; Vinten-Johansen, 2000). Peroxynitrite rapidly isomerises to form inactive nitrate (Eqn 6; Saran *et al.*, 1990), but is also a potent nitrating species, especially direct nitration of tyrosine residues, and oxidising agent, through an alternative decomposition to hydroxyl radical (OH) and nitrogen dioxide (Eqn 7; White *et al.*, 1994; Beckman & Koppenol, 1996; Patel *et al.*, 1999; Vinten-Johansen, 2000).

$$NO + O_2^- \rightarrow ONOO^- \quad (5)$$

$$ONOO^- \rightarrow NO_3^- \quad (6)$$

$$ONOO^- \rightarrow NO_2 + OH \quad (7)$$

1.5.3 Reaction with haem

As mentioned above, NO regulates the activity of sGC through binding to the Fe^{2+} of the haem moiety. This reaction is incredibly rapid, which allows NO to modulate sGC activity at the low concentrations of NO produced by the endothelium, even after allowing for loss of NO in transit (Beckman & Koppenol, 1996). Haem groups are present in a number of other cellular enzymes. NO binds to the haem group of deoxyhaemoglobin (k = 10^{-7} M^{-1}s^{-1}; Gladwin *et al.*, 2000), effectively competing with oxygen. NO also binds to the haem group of NOS, inhibiting NO generation and,

therefore, forming a negative feedback pathway (Buga *et al.*, 1993). NO has also been shown to regulate the function of other haem-containing proteins such as the cytochrome cP450 reductase isozymes (Stamler *et al.*, 1992; Wink *et al.*, 1993), cytochrome oxidases (Brown, 1995; Giulivi, 1998) and catalase (Wink *et al.*, 1993; Brown, 1995). NO can also bind to, and regulate the function of, non-haem iron-containing proteins (Butler *et al.*, 1995; Butler & Megson, 2002).

1.5.4 Reaction with lipoproteins

The plasma membrane is rich in lipids and lipoproteins, which are susceptible to peroxidation. The oxidation of lipids to form lipid peroxyl radicals, may exacerbate or even cause, cardiovascular conditions such as atherosclerosis (Ross, 1993; Maxwell & Lip, 1997). NO itself does not cause lipid peroxidation (Rubbo *et al.*, 1994), but higher nitrogen oxides, such as $NO_2^{\cdot}$, can modify lipids. Indeed, it has been suggested that the reaction of NO with O_2 (to yield higher nitrogen oxides) is greater in the lipid rich compartments of the plasma membrane (Liu *et al.*, 1998). Alternatively, NO may limit lipid peroxidation by directly reacting with lipid peroxyl radicals; essentially acting as an antioxidant (Rubbo *et al.*, 1994; Patel *et al.*, 1999).

1.5.5 Reaction with thiols

A thiol (RSH) is a compound which contains a sulphydryl (SH) group. The group can be S-nitrosated to produce an S-nitrosothiol. S-nitrosothiols share many of the properties of NO and are the focus of much research, including this thesis (RSNOs are discussed later in Sect 1.8.6). The direct reaction of NO with thiol groups is negligible under physiological conditions. However, RSNOs are found physiologically and may serve to 'stabilise' free NO (Stamler *et al.*, 1992; Butler *et al.*, 1995; Gaston, 1999). Formation of S-nitrosothiols is due to the presence of several intermediate reactions.

Firstly, higher nitrogen oxides (nitrogen dioxide, NO_2; dinitrogen trioxide, N_2O_3; dinitrogen tetroxide, N_2O_4), formed from the reaction of NO with O_2, can nitrosate thiols (Eqns 8, 9; Kharitonov *et al.*, 1995; Keshive *et al.*, 1996).

$$2NO_2 + RSH \rightarrow RSNO + NO_3^- + H^+ \quad (8)$$

$$N_2O_3 + RSH \rightarrow RSNO + NO_2^- + H^+ \quad (9)$$

Additionally, peroxynitrite can also react with thiols to form RSNOs, although it is a relatively weak nitrosating agent and is more likely to oxidise thiols to their disuphide (Wink *et al.*, 1994; Mayer *et al.*, 1998). Other redox forms of NO (NO^-, NO^+) will also be able to react with thiols to form RSNOs (Butler *et al.*, 1995; Dierks & Burstyn, 1996). Nitrosonium ions (NO^+) cannot exist in aqueous solution at physiological pH for a significant period of time and therefore NO^+ donors must directly interact with thiol groups to induce nitrosation. The physiological role of nitroxyl ions (NO^-), especially its generation by NOS, is hotly debated.

1.6 NO IN PHYSIOLOGY

Although, this thesis focuses on the role of NO in the cardiovascular system, NO is synthesised in many organs of the body, where it regulates a wide range of essential functions.

1.6.1 Blood vessels

As mentioned previously, NO is synthesised continuously from the endothelium of blood vessels and this basal release contributes to the vascular tone of underlying smooth muscle cells (Palmer *et al.*, 1987; Palmer *et al.*, 1988; Palmer & Moncada, 1989). Subsequently, NO production is now recognised to be a major factor in the control of blood pressure and local blood flow in animals (Aisaka *et al.*, 1989; Rees *et*

al., 1989; Gardiner *et al.*, 1990; Chu *et al.*, 1991) and man (Vallance *et al.*, 1989; Haynes *et al.*, 1993).

1.6.2 Platelets

The endothelium acts as an anti-thrombogenic lining to blood vessels, both physically and through the production of NO. NO donor drugs were first shown to inhibit platelet aggregation in response to ADP, before EDRF was identified as NO (Mellion *et al.*, 1981). Prostacyclin (PGI_2), another endothelium-derived vasodilator, also inhibits platelet aggregation, acting synergistically with NO (Radomski *et al.*, 1987). However, PGI_2 only has weak effects on platelet adhesion (Higgs *et al.*, 1978). NO inhibits the activation of platelets, preventing both aggregation and adhesion to reactive components of the vessel wall (Radomski *et al.*, 1987; May *et al.*, 1991). The mechanism of NO-mediated platelet inactivity is complicated and multifactorial, with both cGMP-dependent and independent mechanisms involved (Gordge *et al.*, 1996; Gordge *et al.*, 1998; Sogo *et al.*, 2000). Platelets themselves synthesise NO via eNOS (Radomski *et al.*, 1990; Radomski *et al.*, 1990) and have been shown to store NO in vesicles as S-nitrosothiols (Hirayama *et al.*, 1999). Synthesis and release of NO (or NO-adducts) occurs when platelets are activated (due to increased intracellular calcium (Ware *et al.*, 1986) and likely acts as a negative feedback to limit the extent of the thrombotic response and provide a means of breakdown of aggregates that has already formed.

1.6.3 Immune cells

NO also inhibits the activity of white blood cells, preventing their activation and adhesion to the vessel wall (May *et al.*, 1991). White cells can synthesise NO following activation by lipopolysaccharide or inflammatory cytokines such as tumour

necrosis factor, interleukins and interferon-γ (Hibbs *et al.*, 1987; Vallance & Moncada, 1994). Activation leads to the expression of iNOS, generating high concentrations of NO (Nussler & Billiar, 1993), that are cytotoxic through inhibition of a number of essential cellular mechanisms, including mitochondrial respiration (Granger & Lehninger, 1982), the citric acid cycle (Hibbs *et al.*, 1988) and DNA replication (Krahenbuhl, 1980). Superoxide may also be generated by white cells, which will subsequently lead to peroxynitrite production (Beckman & Koppenol, 1996). The toxic cocktail of NO, superoxide and peroxynitrite has been suggested to act as a cellular defence mechanism, by killing invading pathogens. Indeed, inhibitors of NOS have been shown to inhibit the cytotoxicity of activated macrophages (Hibbs *et al.*, 1987).

1.6.4 Heart

NO will clearly influence the function of the heart through vasodilatation of blood vessels, reducing pre- and after-load and through dilatation of coronary blood vessels. A number of studies have shown that the endocardium, myocardium and papillary muscles of the heart synthesise NO via eNOS (Schulz *et al.*, 1991; Henderson *et al.*, 1992; De Belder *et al.*, 1993). The stretch of the walls of the heart as it fills with blood releases NO from the endocardium and the endothelium of coronary blood vessels (Smith *et al.*, 1991; Pinsky *et al.*, 1997). The physiological significance of endocardium-derived NO is far from clear (Paulus & Shah, 1999); NO has been suggested to have a positive (Klabunde *et al.*, 1991) or negative (Mohan *et al.*, 1996) inotropic effect, depending on its concentration. NO also alters the contractile state of the myocardium in both systole and diastole (Paulus & Shah, 1999). Additionally, NO regulates the sympathetic nerves that control the heart (Balligand *et al.*, 1993; Bartunek *et al.*, 1997). Conflicting findings of the direct role of NO in the heart may be due to

the high intracellular concentrations of myoglobin (~200 μM) that will presumably scavenge low concentrations of NO in a similar fashion to Hb (Wegener *et al.*, 1999).

1.6.5 Peripheral nerves

The function of NO-releasing nerves was studied before EDRF was identified as NO. Non-adrenergic, non-cholinergic (NANC) nerves synthesise and release an EDRF-like substance, presumably NO or an NO-adduct (Gillespie *et al.*, 1989; Gillespie & Sheng, 1990). Therefore, NO fulfills some of the criteria of a neurotransmitter. NANC nerves regulate the tone of specific blood vessels, including cerebral arteries (Toda & Okamura, 1990) and arteries in the corpus cavernosum that mediate penile erection (Rajfer *et al.*, 1992). They also regulate non-vascular smooth muscle, contributing to bronchodilatation (Barnes, 1993), stomach distension and relaxation of the sphincters of the gut (Burleigh, 1992).

1.6.6 Central nervous system

NOS-containing nerves are widespread throughout the brain. Activation of the neural glutamate receptors, lead to the release of NO (Garthwaite *et al.*, 1988) which may act as a retrograde messenger, sending signals back to pre-synaptic neurons (Vallance & Moncada, 1994). Indeed, S-nitrosation of the glutamate receptor inhibits activity, operating as a negative feedback mechanism (Lipton *et al.*, 1993). NO has also been suggested to be involved in a number of central processes, including arousal (Bagetta *et al.*, 1993), pain perception (Meller & Gerbhart, 1993), memory (O'Dell *et al.*, 1991) and neural degeneration (Rothman & Olney, 1987).

Recently, it has been shown that haemodynamic effects of S-nitrosothiols are altered in the presence of anaesthesia (Travis *et al.*, 1997). Further work showed that direct injection of S-nitroso-L-cysteine into the brain of conscious animals produce a

greater peripheral haemodynamic effect than its stereoisomer S-nitroso-D-cysteine (Davisson *et al.*, 1997). The authors suggest that the central nervous system may contain specific S-nitrosothiol receptors, through which these compounds can exert an influence without prior decomposition to NO.

1.6.7 Kidney

The kidney is a highly important organ, filtering blood and controlling the excretion of metabolites, water and salts. Functional impairment of the kidney has been suggested to be the underlying cause of essential hypertension and will exacerbate a number of other cardiovascular diseases (Guyton *et al.*, 1990; Lifton, 1996). Unsurprisingly, NO from the endothelium of renal blood vessels, regulates blood flow through the kidney and, subsequently, influences sodium homeostasis (Zou & Cowley, 1997; Plato & Garvin, 1999). In addition, NO regulates the release of renin from the kidney (Vidal *et al.*, 1988) and protects against ischaemia-reperfusion injury (Pararajasingam *et al.*, 2000). Many of the effects of NO in the kidney are initiated through activation of the endothelin system, producing a further level of complexity between autocrine and paracrine systems (Plato & Garvin, 1999; Kotelevtsev & Webb, 2001).

1.6.8 Others

NO also exerts a profound effect on the respiratory system. Generation of NO in pulmonary blood vessels modulates basal tone (Greenberg *et al.*, 1987) and, in combination with NANC nerve-derived NO, influences the tone of bronchial smooth muscle. The close interaction between blood vessels and the lung provides a subtle control of oxygen delivery to the blood and inhaled NO has dramatic effects on vessel tone and blood oxygen content (Barnes, 1993). Additionally, NO from immune cells in the lungs will provide an early defence against inhaled pathogens (Barnes, 1993).

As mentioned above, NANC nerves also play a role in gut motility in the gastrointestinal tract. In addition, bacteria in the saliva are involved in the reduction of nitrate, present in many foods, to nitrite. Nitrite is acidified to NO in the stomach and has been suggested to act as a defence mechanism against ingested pathogens (Benjamin *et al.*, 1994). NO may also have a cytoprotective role in the gastrointestinal system through enhanced mucosal blood flow, stimulation of mucus secretion and inhibition of proinflammatory cells (Del Soldato *et al.*, 1999; Bandarage *et al.*, 2000).

Oestrogens regulate eNOS expression and activity and may contribute to the many physiological changes experienced throughout pregnancy (Conrad *et al.*, 1993). NO also contributes to uterine relaxation and vasodilatation during **labour**: a process which is sometimes compared to an 'inflammatory event'. Indeed, there is marked expression of Ca^{2+}-independent NOS before labour, that diminishes after birth (Natuzzi *et al.*, 1993).

1.7 NO IN PATHOPHYSIOLOGY

1.7.1 Overproduction of NO

Most cardiovascular conditions focus on a depletion of endogenous NO. However, sometimes an overproduction of NO, due to iNOS expression can be detrimental.

1.7.1.1 Immune response

As mentioned previously (Hibbs *et al.*, 1987; Vallance & Moncada, 1994), activated white blood cells produce NO as a defence against invading pathogens. However, the production of NO contributes to the inflammatory response, enhancing vasodilatation, vascular leakage and tissue damage (Vallance & Moncada, 1994). NO is generated from iNOS expression in infected tissue cells as well as invading immune cells (Quinn

et al., 1995). iNOS has been detected in the arthritic joints (Farrell *et al.*, 1992), infected kidney nephrons glomeruli (Jansen *et al.*, 1994), psoriatic lesions (Bruch Gerharz *et al.*, 1996), gut ulcers (Middleton *et al.*, 1993) and the liver cells infected with malarial schizonts (Nussler & Billiar, 1993; Morris & Billiar, 1994). In serious infections such as septic shock, there is a massive overactivity of the immune system. Enough NO is generated to have systemic effects, causing potentially fatal hypotension, myocardial depression and non-specific tissue damage (Rees *et al.*, 1990). NOS-inhibitors can reverse this hypotension, but also inhibit constitutive NO generation (Kilbourn *et al.*, 1990; Nava *et al.*, 1991). The development of iNOS-specific inhibitors is likely to be therapeutically advantageous in such conditions (Quinn *et al.*, 1995; Hobbs *et al.*, 1999). However, Bhagat et al. recently showed that iNOS expression may not be responsible for the dilatation of human hand veins in response to cytokines (Bhagat *et al.*, 1999). Instead, the authors propose that there is increased expression of enzymes controlling BH_4 synthesis. Subsequently, enhanced eNOS activation may be the cause of excessive NO generation in sepsis, rather than expression of iNOS.

1.7.1.2 iNOS in cardiovascular disease

In general, there is a decreased bioavailability of NO in cardiovascular diseases (see below). However, iNOS expression is often seen in diseased tissue. In patients with heart disease, iNOS is found in areas surrounding infarcted myocardium (De Belder *et al.*, 1993; Fukuchi *et al.*, 1998; Vejlstrup *et al.*, 1998). iNOS is also found in skeletal muscle of patients with chronic heart failure (CHF; Adams *et al.*, 1997) and in blood vessels of a rat model of CHF (Miller *et al.*, 2000). iNOS is also upregulated in atherosclerotic vessels (Bult *et al.*, 1999; Li & Forstermann, 2000) or normal vessels after vascular injury (Kibbe *et al.*, 1999). iNOS expression may be a counter-mechanism to supplement NO production in areas where constitutive NOS activity is

diminished. Alternatively, it may be acting to counteract an upregulation of vasoconstrictive factors or to negate the effect of NO inactivation by oxygen free radicals. Although, NO may 'scavenge' reactive oxygen species, the formation of peroxynitrite may further accentuate tissue damage and inflammation (White *et al.*, 1994; Beckman & Koppenol, 1996; Vinten-Johansen, 2000). Recently, expression of a dysfunctional NOS isoform, that generates superoxide rather than NO, has been demonstrated in a number of cardiovascular conditions, such as hyperlipidemia (Pritchard *et al.*, 1995), coronary artery disease (Cosentino & Katusic, 1995), nitrate tolerance (Kaesemeyer *et al.*, 2000; Munzel *et al.*, 2000) and heart failure (Miller *et al.*, 2000). This failed counter-regulatory mechanism will further accelerate disease progression.

1.7.2 Endothelial dysfunction in cardiovascular disease

Endothelial dysfunction is characterised as a reduction in the bioavailability of NO and is prevalent in most cardiovascular disorders including hypertension (Linder *et al.*, 1990; Calver *et al.*, 1992; Panza *et al.*, 1993), hypercholesterolaemia (Drexler *et al.*, 1991; Creager *et al.*, 1992), atherosclerosis (Ludmer *et al.*, 1986; Forstermann *et al.*, 1988), heart failure (Kubo *et al.*, 1991; Katz *et al.*, 1992), thrombosis (Loscalzo, 2001) and diabetes (Calver *et al.*, 1992). Originally, endothelial dysfunction was characterised by a reduced response to endothelial-dependent dilators such as acetylcholine or NOS inhibitors like L-NMMA, but not endothelium-independent mediators such as the NO donors sodium nitroprusside or glyceryl trinitrate (Drexler *et al.*, 1991; Calver *et al.*, 1992). Such results were interpreted to be indicative of a reduced production of NO from the endothelium. However, although this interpretation is still likely to be correct, as our understanding of cardiovascular disease grows, it has become clear that endothelial dysfunction is multifactorial (Harrison, 1997; Vallance & Chan, 2001). NO generation by eNOS may be lost due to loss of

endothelial cells, lack of NOS substrate or co-factor availability, or downregulation of eNOS expression (Cohen *et al.*, 1988; Shimokawa & Vanhoutte, 1989; Drexler *et al.*, 1991). An upregulation in the levels of the endogenous NOS inhibitor ADMA has also been found in hypertensive patients with renal failure (Vallance *et al.*, 1992), hypercholesterolaemic rabbits (Yu *et al.*, 1994) and endothelial cells that have regenerated following denudation by balloon angioplasty (Azuma *et al.*, 1995). Increases in oxidized lipoproteins may also enhance eNOS dysfunction and NO diffusion (Galle *et al.*, 1991; Tanner *et al.*, 1991; Pritchard *et al.*, 1995). Available NO can be further diminished by reactive oxygen species-mediated scavenging under conditions of oxidative stress brought on by increased oxygen radical production or reduced anti-oxidant protection (Gryglewski *et al.*, 1986; Nakazono *et al.*, 1991). Finally, it should be highlighted that vascular smooth muscle itself may become less sensitive to NO cardiovascular diseases (Robinson *et al.*, 1982; Calver *et al.*, 1992), although the exact cause(s) have not been fully elucidated.

In more severe cardiovascular disease, vascular remodelling and ventricular hypertrophy increases the number of cells and distance NO must diffuse to cause relaxation of the outermost VSMCs and myocytes (Egan *et al.*, 1987; Folkow, 1990). Additionally, disruption to laminar blood flow can reduce eNOS activity and reduce the proximity of NO-scavenging red blood cells to the endothelium (Wei Liu *et al.*, 1989; Butler *et al.*, 1998).

1.7.3 Vascular disease and vessel occlusion

Vascular disease, or atherosclerosis, is a common condition seen in aging blood vessels, particularly in patients who have a number of risk factors including diabetes, smoking, high cholesterol levels and hypertension (Vogel, 1997). At present it is unclear if endothelial dysfunction is a cause or result of early stages of atherosclerosis, although the loss of the protective effects of NO clearly worsens the situation

(Celermayer *et al.*, 1992). The initial marker of vascular disease is the deposition of lipids, forming a 'fatty streak'. As the disease progresses, a distinct lesion begins to form, composed of many cells including proliferating smooth muscle cells, white-blood cell derived foam cells and fat and calcium deposition (Ross, 1993; Davies, 2000). The disease can progress further until a distinctly structured atherosclerotic lesion forms and begins to protrude into the the vessel lumen, obstructing flow. Areas of the lesion may become weakened, exposing reactive components, leading to thrombus formation which further occludes the lumen. Sudden plaque rupture will lead to a massive area of thrombosis and inflammation at the exposed lipid core, that may totally occlude the vessel lumen. Even 'stable plaques' are vunerable to endothelial erosion that leads to chronic thrombosis at these sites (Davies, 2000). Aggregates may break free from the plaque surface, forming circulating microemboli that may lodge in small vessels elsewhere in the circulation. In the case of the coronary circulation, when blood flow through arteries is diminished to such an extent that the heart no longer receives adequate blood to maintain function, ischaemia will occur and the area of the heart become infarcted (Opie, 1990). Infarction of the heart worsens the efficiency of the heart, exacerbating the situation, until the heart fails. It has been estimated that acute myocardial infarction due to vascular occlusion accounts for 250,000 deaths each year in the UK (Gershlick, 2001).

1.7.4 Non-pharmacological intervention

A number of surgical and cardiological techniques have been developed to restore flow through coronary blood vessels.

1.7.4.1 Bypass grafting

Coronary artery bypass grafting (CABG) is an invasive surgical technique used when there is extensive multiple vessel disease in the coronary circulation (Kaiser, 1985). Essentially a blood vessel is surgically grafted or rerouted to improve blood flow to ischaemic regions. A blood vessel, such as a saphenous vein or radial artery, is isolated and then grafted from a patent artery to an artery in the ischaemic region, 'bypassing' the obstruction. Alternatively, the internal mammary artery may be redirected to the ischaemic region, without directly transplanting a blood vessel from elsewhere in the body. Open heart surgery clearly carries a great deal of risk, but the technique has been shown to improve heart function and reduce cardiovascular events compared to medicinal therapy alone (Berger *et al.*, 2001; Hueb *et al.*, 2001). However, long-term success is limited by spasm, accelerated atherosclosis and thrombolytic occlusion of the graft, and high dose thrombolytic therapy has complications preventing long-term use (Janero & Ewing, 2000).

1.7.4.2 Angioplasty

The prevalence of vascular diseases encouraged researchers to develop a less invasive technique to recanalise occluded coronary arteries. In the 1970s Gruntzig *et al.* modified balloon catheters, previously used to restored blood flow through peripheral arteries, to allow them to be used in the coronary circulation (Gruntzig *et al.*, 1979). Essentially a catheter with a deflated balloon at its tip is introduced into the femoral artery under local anaesthetic. The catheter is manipulated into the coronary circulation via shaped guidewires. The balloon is placed at the site of the obstruction and then inflated. Inflation stretches the artery, 'cracks' the lesion and, subsequently, restores blood flow (Janero & Ewing, 2000; Fig 1.9). Increasing expertise and catheter design improved the success rate, in terms of improved blood flow, of percutaneous transluminal coronary angioplasty (PTCA) to ~90%. Clinical trials showed that PTCA

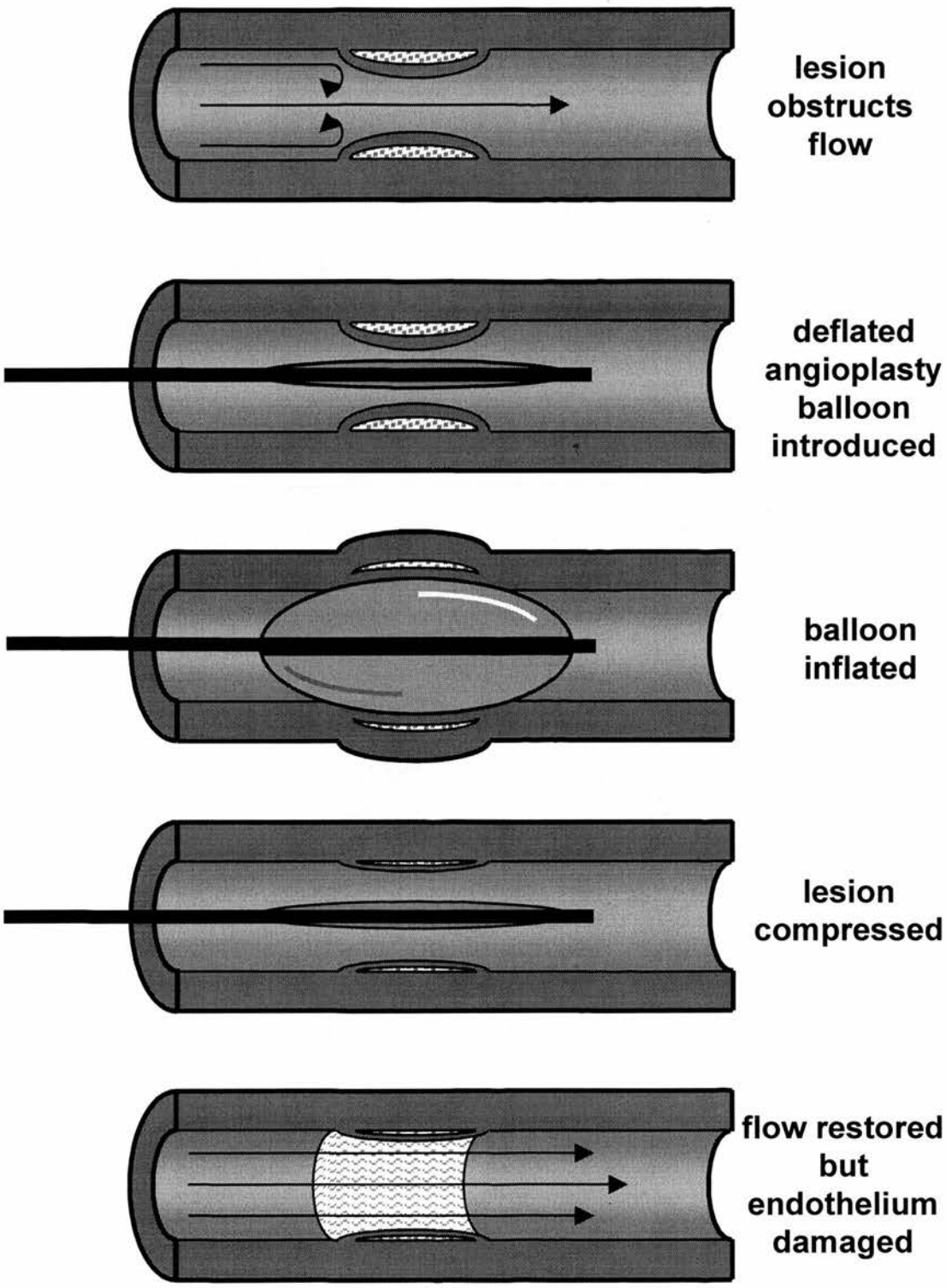


Figure 1.9 Schematic diagram of the angioplasty procedure, used to improve blood flow through an obstructed artery.

was as efficient as CABG in practically all subgroups of patients with vascular disease (Berger *et al.*, 2001; Gershlick, 2001).

The main limitation of CABG and PTCA is the reocclusion of treated arteries due to acute thrombosis and vasospasm (Mabin *et al.*, 1985). In PTCA, in particular, these processes lead to chronic reocclusion due to restenosis. Restenosis is multifactorial and involves elastic recoil, adhesion of blood cells to artery, proliferation and migration of intimal smooth muscle cells and vascular remodelling (Wei Liu *et al.*, 1989; Bult, 2000; Janero & Ewing, 2000). Damage to the endothelium of treated arteries is unavoidable and will contribute to most of the above processes. Despite advances in pharmacological agents (Swanson *et al.*, 2001) and drug delivery systems (Lincoff *et al.*, 1994; Wolinsky, 1994), restenosis still remains a problem. In fact, >30% of patients require additional intervention within 6 months after angioplasty (King *et al.*, 1994), inflicting a huge burden on the healthcare budget (Wei Liu *et al.*, 1989; Lincoff *et al.*, 1994; Janero & Ewing, 2000). Angioplasty will be discussed further in Chapter 7.

1.7.4.3 Stenting

A further recent adaptation to angioplasty has been the deployment of stents (see Ruygrok & Serruys, 1996, for history), which are now in routine use (Topol & Serruys, 1998; Fleisch & Meier, 1999). Stents are small metal devices, formed of a mesh of struts that fit over the deflated angioplasty balloon. When the balloon is inflated, the stent expands, forming a tubular structure that holds the vessel wall open. The rigid structure of the stent prevents elastic recoil and vasospasm, maintaining patency and restricting the extent of vascular remodelling. However, the metallic nature of stents themselves promotes causing smooth muscle cells of the intima to proliferate extensively (Hoffmann *et al.*, 1996) and neointimal size is often greater than that associated with angioplasty alone. Consequently, restenosis still remains a problem in

over 20% of cases (Fischman *et al.*, 1994; Serruys *et al.*, 1994; Bauters *et al.*, 1998). Antiplatelet agents, such as aspirin, clopidogrel and gycoprotein (GP) IIb/IIIa inhibitors and the coating of stents with non-reactive, or drug eluting, surfaces has had a reasonable degree of success in reducing neointima formation (Swanson *et al.*, 2001). For example, stents eluting the anti-mitotic compound sirolimus (rapamycin) have been particularly effective at inhibiting neointimal hyperplasia (Sousa *et al.*, 2001). However, these compounds do not have anti-platelet actions and the long-term effectiveness of such stents (> 1 yr) has yet to be established. At present, restenosis still remains a significant problem (Bult, 2000; Swanson *et al.*, 2001).

1.8 NO DONORS

Due to the prevalence of endothelial dysfunction in cardiovascular disease and unavoidable damage following interventional cardiology, the delivery of exogenous NO to areas of diminished NO activity is an attractive therapeutic option in the management of these conditions. There is now an extensive number of classes of NO donors, yet only the organic nitrates and sodium nitroprusside (SNP) are used clinically and both have limitations. With the development of novel NO donors, NO supplementation is likely to become more prevalent for a wider range of disorders (Megson, 2000).

1.8.1 Organic nitrates

1.8.1.1 Mechanism of action

Organic nitrates, particularly glyceryl trinitrate (GTN; Fig 1.10), have been used medicinally since the 19th century. They are still the most commonly used NO donors

today, mainly to relieve the symptoms of angina. Nitrates are recognized to be venoselective (MacAllister *et al.*, 1995) and their symptomatic effect is primarily due to decreased venous return to the heart, reducing cardiac workload. The dilatation of large coronary and collateral arteries will also enhance their effectiveness (Parker, 1987; Bauer *et al.*, 1995). Despite their frequent use, their mechanism of action is poorly understood.

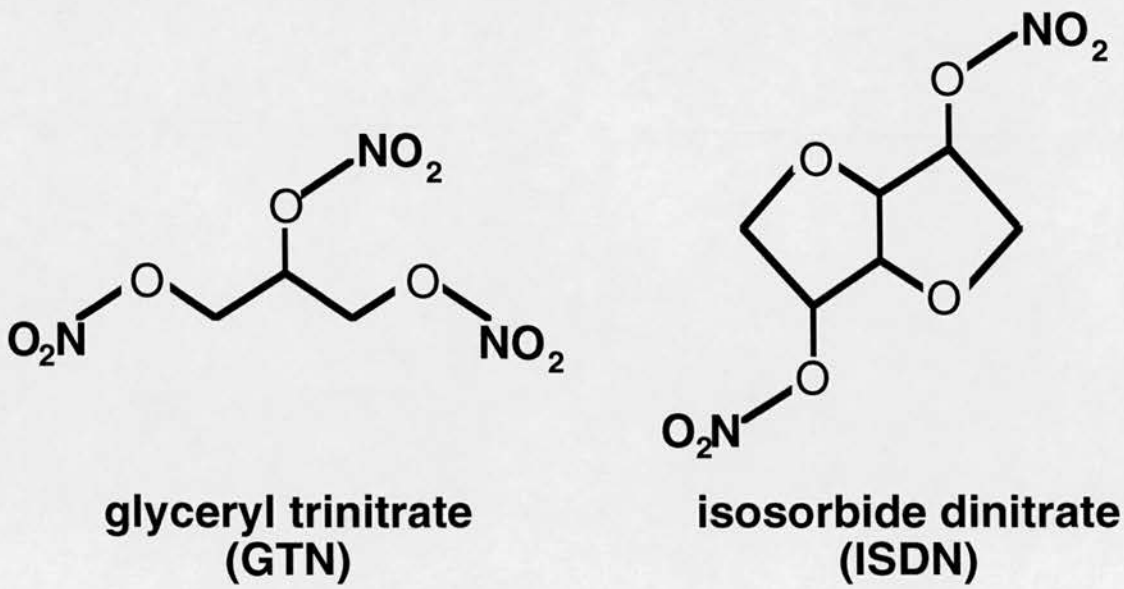


Figure 1.10 Chemical structure of two organic nitrates, GTN and ISDN.

GTN is particularly stable in comparison to other NO donors and it does not generate NO spontaneously in solution. Instead GTN must be biotransformed to active NO. In VSMCs, 1 mole of NO is generated per molecule of GTN (Bennett *et al.*, 1989). The factor that biotransforms GTN *in vivo* remains elusive. Initially, studies focused on reducing agents, such as thiols. Thiols were proposed to reduce the nitrate groups to nitrite and then further react with nitrite to generate free NO or unstable S-nitrosothiols (Ignarro *et al.*, 1981). There is an overwhelming body of contradictory work in this area (Fung *et al.*, 1989), but the consensus is that thiols are unlikely to be sufficiently powerful reducing agents to facilitate all the reduction steps needed to metabolise GTN

to NO (Megson, 2000; Schroder, 1985), even at the high physiological concentrations of intracellular GSH in blood vessels (0.5-10 mM; Boesgaard *et al.*, 1993; Kurz *et al.*, 1993; Haj-yehia & Benet, 1996).

The observation that the stereoisomers of the nitrate isosorbide dinitrate (ISDN; Fig 1.10) have a 10-fold difference in potency in blood vessels (Bennett *et al.*, 1988) suggests that biological enzymes mediate biotransformation to NO. Initially, glutathione-S-transferases (GSTs) were suggested to cleavage of nitrate groups, via a glutathione-dependent mechanism. Results were once again conflicting (Yeates *et al.*, 1989; Sakanashi *et al.*, 1991; Chung *et al.*, 1992; Kurz *et al.*, 1993; Seth & Fung, 1993). GSTs can indeed mediate partial denitrification of nitrates such as GTN, although these enzymes selectively remove the central nitrate, producing 1,3-GDN as a by-product (Jakoby *et al.*, 1976). However, convincing evidence suggests that it is the denitration of one of the terminal nitrates (co-producing of 1,2-GDN) that is linked to the biological activity of GTN (Brien *et al.*, 1986; Bennett *et al.*, 1989).

A better, but far from definite, proposal is that the cytochrome P450 pathway mediates biotransformation (Servent *et al.*, 1989; Schroder & Schror, 1990; Yeates, 1992; Minamiyama *et al.*, 2001). A 200 kD microsomal protein in the membrane of smooth muscle cells has also been identified as the endogenous nitrate bioactivator (Chung & Fung, 1990; Chung & Fung, 1992; Seth & Fung, 1993), but it has yet to be fully isolated and characterised (for review of potential enzymes see Bennett *et al.*, 1994; Bauer *et al.*, 1995).

1.8.1.2 Therapeutic applications

In 1867 amyl nitrite was used to treat angina in Edinburgh (Brunton, 1867), followed 12 years late by the use of the organic nitrate, GTN (Murrell, 1879); a compound that is still in clinical use today. Occasionally, it is also used in the treatment severe myocardial ischaemia and infarction. GTN can be administered as sprays, ointment,

sublingual tablets and slow release GTN patches. Transdermal patches allow the duration of action to be extended up to 8 h to treat patients with chronic angina. Oral ISDN and isosorbide mononitrate are alternatives that have a slower onset, but a longer duration of action (Parker, 1987).

Experimentally, GTN has been investigated in many models of other cardiovascular diseases. Transdermal patches reduce platelet activation (Herbert *et al.*, 1997), despite GTN being a weak inhibitor of platelet aggregation *in vitro* (Sogo *et al.*, 2000). This may be because platelets themselves lack the mechanism to biotransform GTN (Weber *et al.*, 1996), with sufficient biotransformation in vascular tissue *in vivo* to influence platelet activity (Megson & Webb, 2000). This antiplatelet activity contributes to the anti-anginal effects of GTN, by preventing cyclic flow reductions in the coronary circulation (Folts *et al.*, 1991). GTN has also been demonstrated to reduce platelet adhesion in a model of angioplasty (Lam *et al.*, 1988). However, high concentrations are required for sufficient benefit, leading to systemic side-effects such as hypotension. Additionally, GTN was only effective in animals with extensive arterial injury, which occurs infrequently with modern day angioplasty catheters and clinical expertise.

Despite improving heart function, at present it is unclear whether nitrates significantly reduce mortality in patients with myocardial ischaemia/infarction (Yusuf *et al.*, 1988; ESPRIM group, 1994; Munzel, 2001). Other side-effects of organic nitrates are severe headaches, postural hypotension, and the development of tolerance (Parker, 1987).

1.8.1.3 Nitrate tolerance

The major limitation of organic nitrates is the induction of tolerance. Tolerance is defined as the loss of therapeutic effect with continuous administration; GTN is almost completely ineffective within 24 hours). Although activity can be restored by a 4-12

hour drug free period, intermittent dosing can be a problem for patients with chronic angina (Abrams *et al.*, 1998).

Due to the lack of understanding of the mechanism of organic nitrate bioactivation, it is unsurprising that the underlying cause of tolerance also remains a mystery. Many theories have been proposed, including plasma volume expansion (Parker *et al.*, 1991), upregulation of neurohormonal vasoconstrictor pathways (Packer *et al.*, 1987; Parker *et al.*, 1991; Munzel *et al.*, 1995) and desensitization of sGC (Axelsson & Andersson, 1983; Waldman *et al.*, 1986; Mulsch *et al.*, 2001). Most attention has focused on the hypothesis that tolerance is caused by impaired activation of GTN (Brien *et al.*, 1986; Slack *et al.*, 1989; Feelisch & Kelm, 1991), particularly as levels of 1,2-GDN formation are reduced in tolerant tissues (Bennett *et al.*, 1989). It had previously been shown that alkylation of free thiols inhibited the vasodilatory effect of GTN (Needleman *et al.*, 1973; Needleman & Johnson, 1973) and that nitrate tolerance could be prevented, or partially reversed, by administering exogenous thiols (Packer *et al.*, 1987). For many years it was assumed that depletion of thiols necessary for the biotransformation of nitrates was the underlying cause of tolerance. There is an overwhelming body of literature investigating the role of thiols in tolerance induction (Gruetter & Lemke, 1985; Fung *et al.*, 1989; Munzel *et al.*, 1989; Boesgaard *et al.*, 1993; Haj-yehia & Benet, 1996), although presence of numerous contradictory reports suggest that thiol depletion is not the underlying cause of tolerance, but may be observed as a consequence.

More recently, two novel theories have received much attention (Parker & Gori, 2001). Munzel et al. showed that tolerance was accompanied by an increase in superoxide generation leading to scavenging of GTN-derived NO (Munzel *et al.*, 1995). The authors elaborated further, showing that GTN produced superoxide through stimulation of endothelial NAD(P)H-dependent oxidases (Munzel *et al.*, 1996), decreased levels of SOD (Munzel *et al.*, 1999) and induction of a dysfunctional form of eNOS (Kaesemeyer *et al.*, 2000; Munzel *et al.*, 2000). Upregulation of

oxidant stress can explain the beneficial effects of thiols in tolerance; acting as antioxidants in a similar way to vitamins C (Bassenge *et al.*, 1998; Watanabe *et al.*, 1998) and E (Watanabe *et al.*, 1997). Attention has also returned to cGMP-dependent phosphodiesterases, as there is an upregulation of phosphodiesterase expression and activity in nitrate-tolerant vessels (Axelsson & Andersson, 1983; Kim *et al.*, 2001). However, both novel theories do not sufficiently explain all the contradictory findings (see Chapter 5) and it is likely that we will only understand organic nitrate tolerance when we more fully understand the mechanism of nitrate action.

1.8.2 Sodium nitroprusside (SNP)

1.8.2.1 Mechanisms of action

SNP is a nitrosyl-iron complex containing five cyanide groups (Fig 1.11).

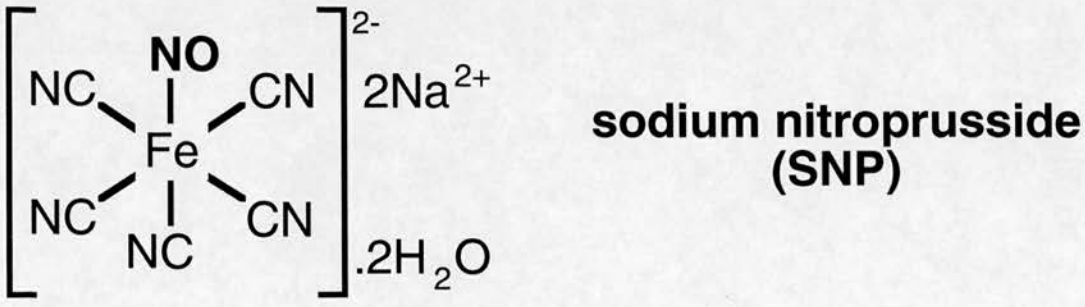


Figure 1.11 Chemical structure of SNP.

The mechanism of SNP biotransformation is, once again, poorly understood. It is often assumed that SNP decomposes to NO spontaneously in solution (Ignarro *et al.*, 1981; Feelisch & Noack, 1987; Schroder & Schror, 1990). However, SNP is stable in solution at physiological pH (Butler & Glidewell, 1987),unless exposed to light, reducing agents including thiols, or biological factors (Ignarro & Gruetter, 1980;

Leeuwenkamp *et al.*, 1986; Bates *et al.*, 1991; Kowaluk *et al.*, 1992; Marks *et al.*, 1995). Similarly to GTN, cytochrome P450 reductase enzymes have also been implicated in the biotransformation of SNP to NO (Rao *et al.*, 1991), although the physiological importance of this has yet to be determined (Smith & Kruszyna, 1974). Additionally, a smaller membrane-bound protein, distinct from the protein that metabolises GTN, has been identified that releases NO from SNP (Kowaluk *et al.*, 1992). Membrane-mediated biotransformation to NO is a possibilty, as nitroprusside is a divalent ion and therefore would be unlikely to cross the plasma membrane (Kowaluk *et al.*, 1992). However, SNP has been shown to cross biological membranes, albeit slowly (Rodkey & Collison, 1977; Butler *et al.*, 1988). Also, the lack of inhibitory effect of membrane-impermanent haemoproteins on SNP-induced vasodilatation, suggests an intracellular breakdown (Gruetter *et al.*, 1979). Unlike organic nitrates, SNP is a potent inhibitor of platelet aggregation *in vitro* (Sogo *et al.*, 2000), suggesting that platelets contain factors that can activate SNP. Additionally, SNP is equipotent in arteries and veins (Armstrong *et al.*, 1975) and does not induce self-tolerance or show cross-tolerance to GTN (Kieth *et al.*, 1982; Kowaluk *et al.*, 1987; Hinz & Schroder, 1998; Sage *et al.*, 2000; Minamiyama *et al.*, 2001).

1.8.2.2 Therapeutic applications and limitations

SNP was first used as a vasodilator in 1929. Nowadays, the compound is only occasionally used to control severe hypertension and induce hypotension during anaesthesia (Butler & Glidewell, 1987). SNP needs to be given as an intravenous infusion, limiting its clinical use. Also, it is considered difficult to titrate due to its potent and rapid hypotensive effect and the necessity to be shielded from light during preparation and infusion (Megson, 2000). More worrying is the presence of the five cyanide groups in the molecule, which can be released through interaction with red blood cells (Smith & Kruszyna, 1974). It has been suggested that prior release of

cyanide groups may even be necessary to release NO (Bates *et al.*, 1991), although this is controversial (Butler *et al.*, 1988). Normally, the body can cope with low concentrations (0.5 mg/kg/hr) of cyanide by converting it to thiocyanate via the thiosulphate-dependent rhodanase system, but that said, in isolated instances cyanide poisoning has been seen with prolonged use of SNP (Michenfelder & Tinker, 1977). Nowadays, the use of SNP is mostly restricted to clinical studies, where it is regularly used as an endothelium-independent vasodilator.

1.8.3 Molsidomine derivatives (SIN-1)

1.8.3.1 Mechanisms of action

Molsidomine is converted to the NO donor 3-morpholinosydnonomine (SIN-1 or linsidomine) in the liver *in vivo* (Bohn & Schonafinger, 1989). SIN-1 must undergo several further reactions before NO is finally released and these occur spontaneously in the blood (Feelisch *et al.*, 1989). Firstly, SIN-1 is converted to SIN-1A via hydroxylation. Secondly, molecular oxygen converts SIN-1A to SIN-1C, producing NO as a by-product (Bohn & Schonafinger, 1989; Fig 1.12). Like other NO donors, SIN-1 was orignally believed to act through the stimulation of sGC (Noack & Feelisch, 1989), although Rinaldi *et al.* (Rinaldi & Cingolani, 1983) demonstrated a poor association between the time course of cGMP generation and haemodynamic effect, suggesting that other cellular mechanism are involved. Unlike organic nitrates, SIN-1 has anti-platelet actions (Gerzer *et al.*, 1989) and does not induce self-tolerance or exhibit cross-tolerance with nitrates (Sutsch *et al.*, 1989; Rudolph & Dirschinger, 1991; Hinz & Schroder, 1998).

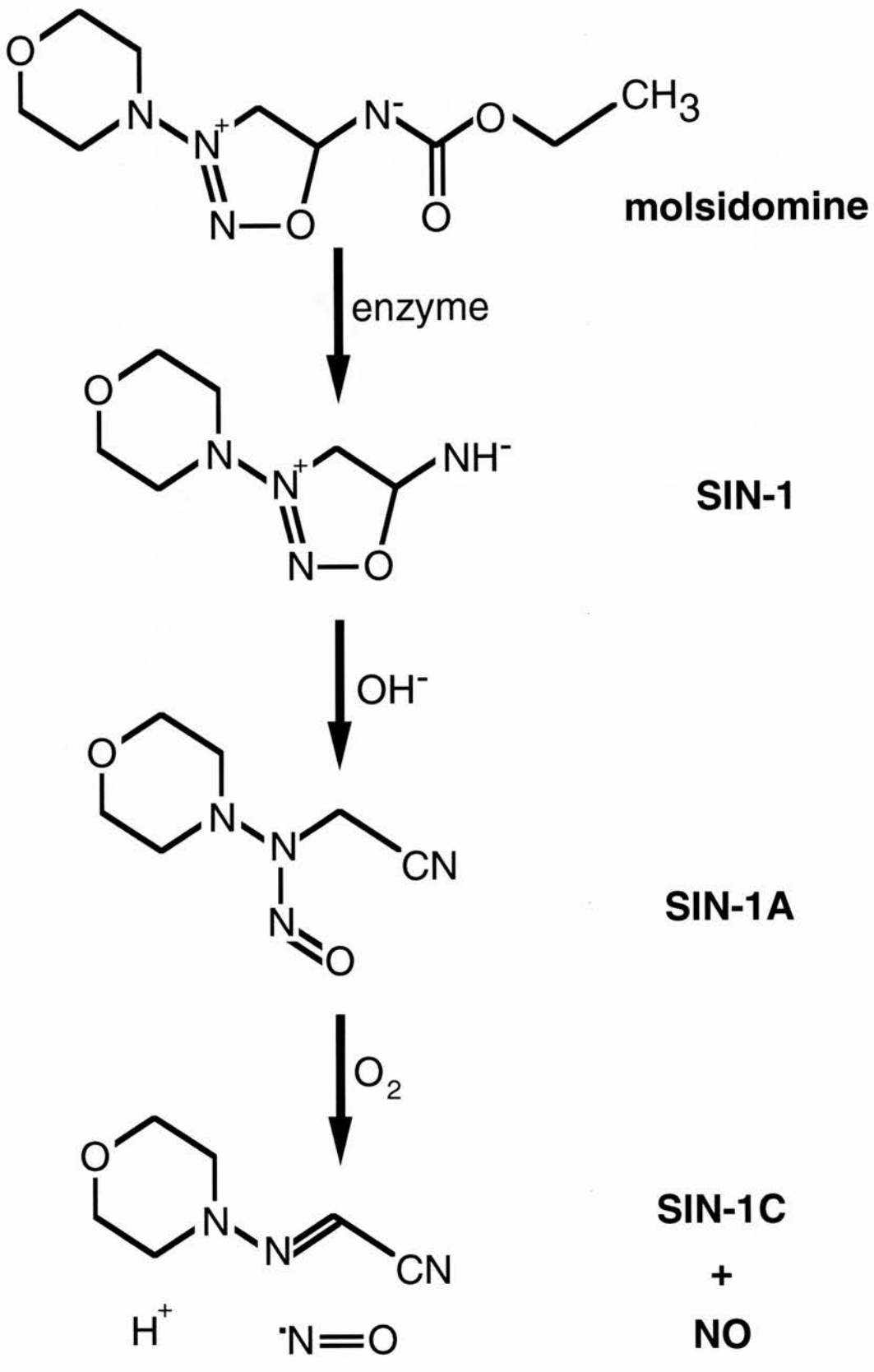


Figure 1.12 Pathway of NO generation from molsidomine via SIN-1.

1.8.3.2 Therapeutic applications and limitations

Molsidomine derivatives were first used in the treatment of angina in the late 1970s (Majid *et al.*, 1980) and have subsequently been used clinically to treat coronary vasospasm (Danchin *et al.*, 1991), heart failure (Unger *et al.*, 1994) and angina (Messin *et al.*, 1995). Molsidomine, but not its derivatives, can be administered orally.

Experimentally, molsidomine and SIN-1 have been shown to reduce platelet adhesion to vessels following balloon angioplasty. A reduction in the extent and prevalence of thrombotic occlusion was shown (Groves *et al.*, 1993; Provost *et al.*, 1997), but there was variability in the effectiveness of the compounds to prevent restenosis (Groves *et al.*, 1995; Rikitake *et al.*, 1998; Kalinowski *et al.*, 2001). That said, the ACCORD study, a large clinical trial, also showed improved luminal diameter 6 months after coronary angioplasty (Lablanche *et al.*, 1997). Results from other experimental models show that SIN-1 has other anti-atherogenic effects, by inhibiting the oxidation of lipoproteins (Rikitake *et al.*, 1998) and the proliferation of smooth muscle cells (Groves *et al.*, 1995).

However, the therapeutic potential of SIN-1 is limited by findings that it co-generates superoxide (Feelisch *et al.*, 1989; Holm *et al.*, 1998). Superoxide itself will have a detrimental effect, but localised and simultaneous generation of NO and superoxide will undoubtedly produce cytotoxic peroxynitrite. Indeed, SIN-1 is often used as a peroxynitrite generator in scientific studies (Lipton *et al.*, 1993; Amirmansour *et al.*, 1999; Mathy-Hartert *et al.*, 2000). Peroxynitrite generation may explain the lack of beneficial effects of these compounds in preventing neointimal growth (De Meyer *et al.*, 1995) and in reducing mortality in patients with chronic heart failure (ESPRIM group, 1994) or coronary artery disease (Lablanche *et al.*, 1997).

1.8.4 Diazeniumdiolates (NONOates)

1.8.4.1 Mechanisms of action

Diazeniumdiolates (NONOates) constitute a novel class of NO donor drugs. Although these compounds have been recognised for many years, they became the focus of attention as NO donor drugs in 1991 (Maragos *et al.*, 1991). They are composed of a diolate group ([N(O^-)N=O]) bound to nucleophile adduct (a secondary or primary amine or polyamine) via a nitrogen atom (Maragos *et al.*, 1991). NONOates decompose spontaneously at physiological pH and temperature, to generate up to 2 molar equivalents of NO. The rate of decomposition is dependent on the structure of the nucleophile adduct (Hrabie *et al.*, 1993). A range of NONOates have now been described with half-lives varying from seconds to hours (Morley & Keefer, 1993; Fig 1.13). It has been proposed that the modification of the structure of the nucleophile adduct may also engender selectivity for vascular beds and cellular absorption (Brilli *et al.*, 1997).

An attractive feature of this class of compounds is that decomposition is not catalysed by thiols or biological tissue and can be predicted following first-order kinetics (Morley *et al.*, 1993; Mooradian *et al.*, 1995). Subsequently, biological activity such as vasodilatation (Maragos *et al.*, 1991; Morley *et al.*, 1993), inhibition of platelet aggregation (Diodati *et al.*, 1993; Sogo *et al.*, 2000) and inhibition of VSMC proliferation (Mooradian *et al.*, 1995) closely correlate with the amount of NO generated *in vitro*. Unsurprisingly, NONOates are not susceptible to tolerance, presumably because they do not require a biological factor to generate NO (Hinz & Schroder, 1998).

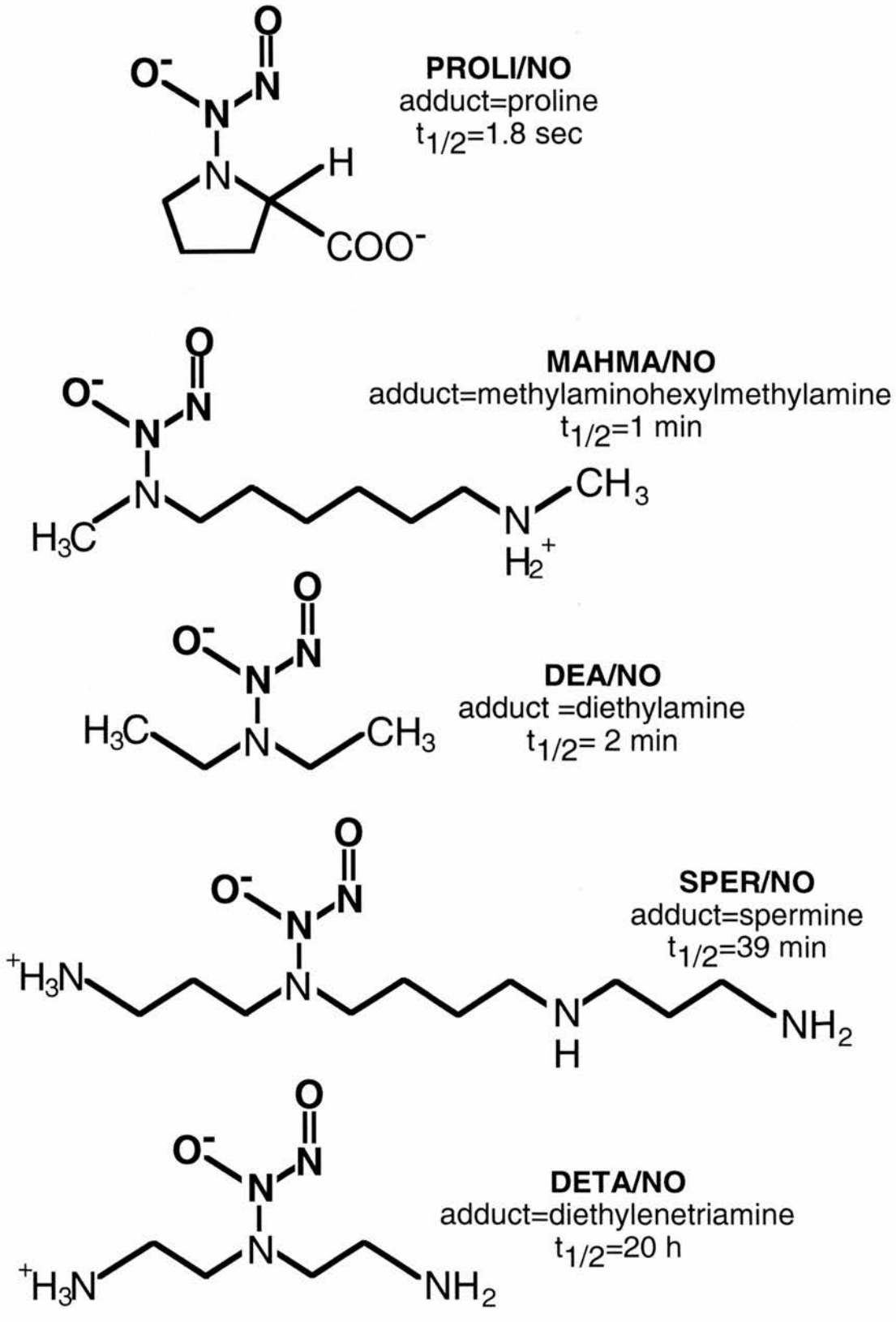


Figure 1.13 Chemical structure of five examples of the NONOate class of NO donors. Note the wide range of half-lives.

1.8.4.2 Therapeutic applications

At present NONOates have not been used clinically and have been tested in surprisingly few animal models of cardiovascular diseases. That said, results from animal models of neointima formation look promising. SPER/NO applied peri-vascularly reduced neointima formation induced by peri-arterial collars (Yin & Dusting, 1997), balloon angioplasty (Kaul *et al.*, 2000) and also in bypass veins (Chaux *et al.*, 1998). MAHMA/NO-eluting stents also reduce platelet adhesion to artificial grafts (Hanson *et al.*, 1995) and local infusion devices releasing PROLI/NO reduced cell proliferation following endarterectomy-induced injury (Chen *et al.*, 1997). Additionally, DEA/NO prevents and reverses vasospasm in a primate model of subarachnoid hemorrhage, without affecting systemic blood pressure (Pluta *et al.*, 1997). Three NONOates have been shown to lower vascular resistance in models of pulmonary hypertension (Vanderford *et al.*, 1994; Brilli *et al.*, 1997). The use of NONOates orally has yet to be fully clarified, although transdermal preparations have already been developed (Shabani *et al.*, 2001).

NONOates will undoubted be investigated in clinical trials once long-term safety has been established. The toxicity of by-products needs to be confirmed, especially whether subsequent reactions between decompostion products leads to the formation of carcinogenic nitrosamines (Maragos *et al.*, 1991). The known rate of NO generation and wide range of half lives are a distinct advantage that could be exploited for medical gain.

1.8.5 Hybrid NO donor drugs

Hybrid NO donors represent a novel approach to the design of NO releasing compounds. This class covers a range of established drugs that have been structurally modified to incorporate NO-containing moieties. The aim of this strategy is to

synthesise drugs that release NO, but still retain the pharmacological activity of the parent compound. Importantly, the release of NO must be balanced to provide sufficient activity within the concentration range of the parent compound (Bandarage *et al.*, 2000).

1.8.5.1 S-Nitrosated adducts

Initially, a hybrid approach was applied to inhibitors of angiotensin converting enzyme (ACE). ACE is the endogenous enzyme which converts angiotensin I to angiotensin II; a potent vasoconstrictor and anti-natriuretic agent. Captopril is an example of an ACE inhibitor that contains a SH group, which can be nitrosated, forming S-nitrosocaptopril (SNO-Cap; Loscalzo *et al.*, 1989; Fig 1.14). SNO-Cap has sGC-mediated vasodilator and anti-platelet action, yet retains the ability to inhibit ACE (Cooke *et al.*, 1989; Loscalzo *et al.*, 1989). Additionally, SNO-Cap appears to preferentially dilate coronary arteries (Cooke *et al.*, 1989). Intravenous SNO-Cap produces a long-lasting hypotensive effect *in vivo* (Shaffer *et al.*, 1991) and, similarly to other S-nitrosothiols, is less susceptible to tolerance (Shaffer *et al.*, 1991; Zhang *et al.*, 1994; Matsumoto *et al.*, 1995). Part of the NO-mediated actions of SNO-Cap may be due to ACE inhibition, as ACE also inactivates bradykinin, an endogenous stimulator of EDRF (Marks *et al.*, 1980).

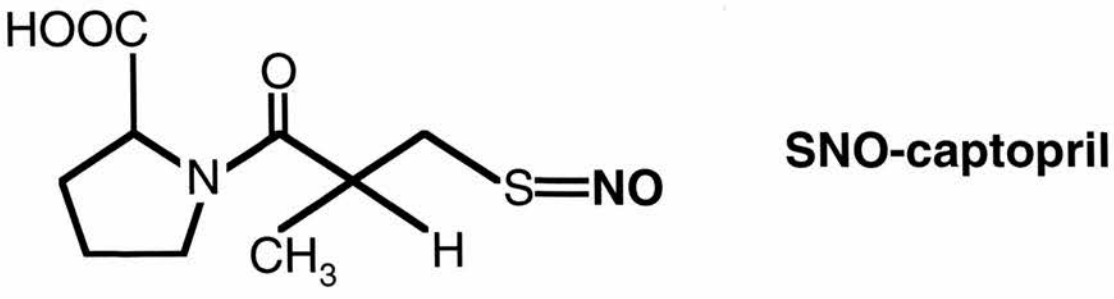


Figure 1.14 Chemical structure of the hybrid NO donor/ACE-inhibitor, SNO-Cap.

Tissue-type plasminogen activator (t-PA) is an endogenous enzyme synthesised by the endothelium. Fibrin, a constituent of thrombus, binds to t-PA stimulating the conversion of plasminogen to plasmin; a powerful fibrinolytic agent. t-PA contains a single SH group which can be S-nitrosated, allowing t-PA to directly inhibit platelets, as well exerting a slightly greater fibrinolytic activity than native t-PA (Stamler *et al.*, 1992). More recently, the combined anti-thrombotic and anti-inflammatory action of SNO-t-PA has been shown to reduce cardiac necrosis following ischaemia-reperfusion injury *in vivo* (Delyani *et al.*, 1996).

Von Willebrand factor (vWF) is synthesised and released by damaged blood vessels. The binding of vWF to platelet glycoprotein receptors induces platelet activation, causing adhesion to the damaged region. Recombinant fragments of vWF, such as AR545C, bind to platelet receptors, competing with the binding of endogenous vWF to platelet receptors and therefore prevent the initiation of thrombosis. Inbal *et al* have shown that S-nitrosated AR545C causes a greater inhibition of platelet adhesion and aggregation than recombinant vWF that is not S-nitrosated (Inbal *et al.*, 1999).

1.8.5.2 NO adducts of non-steroidal anti-inflammatory drugs

A particularly attractive concept is the addition of NO to non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the generation of thromboxanes by inhibition of cyclo-oxygenase, which induce platelet activation. The simultaneous release of NO should act synergistically with the NSAID-adduct, to provide greater anti-platelet activity. The major limitation of NSAIDs are their gastric toxicity due to the inhibition of prostacyclin in the gut.Therefore, another potential benefit of NO-releasing NSAIDS is that NO may alleviate NSAID-induced gastrointestinal irritation by enhancing mucosal blood flow, stimulating mucus secretion and inhibiting gastrointestinal inflammatory cells (Wallace & Cirino, 1994; Del Soldato *et al.*, 1999; Bandarage *et al.*, 2000).

Nitrate groups have been successfully incorporated into the most commonly used NSAID, aspirin (NCX4016, NCX4215; Fig 1.15a). Additionally, an S-nitrosothiol ester can be linked to another frequently used NSAID, diclofenac (Bandarage *et al.*, 2000; Fig 1.15b).

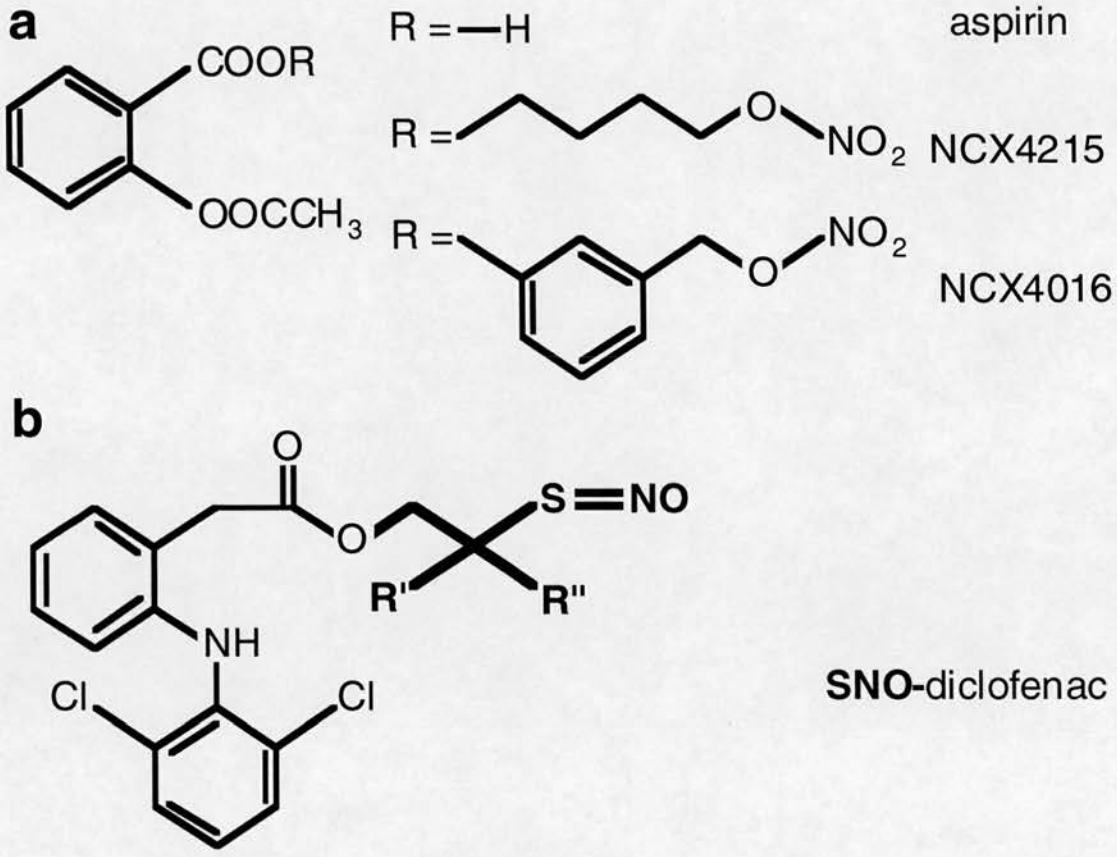


Figure 1.15 Chemical structure of three NO-NSAID hybrids. (a) R = structural changes allowing addition of a nitrate group to aspirin. (b) Area highlighted in bold represents the structural changes facilitating addition of a S-nitrosothiol group to diclofenac.

Several studies show that these compounds have comparable or greater anti-platelet effects than the parent NSAID, without causing excessive vasodilatation or hypotension (Lechi *et al.*, 1996; Del Soldato *et al.*, 1999; Wallace *et al.*, 1999; Momi *et al.*, 2000). NCX-4016 has also been demonstrated to have a greater inhibitory effect on restenosis than aspirin, following PTCA in a transgenic model of hypercholesterolaemia (Napoli *et al.*, 2001). Importantly, these drugs still retain the analgesic and peripheral anti-inflammatory effects of the parent compound, when given orally (Ukawa *et al.*, 1998; Del Soldato *et al.*, 1999; Bandarage *et al.*, 2000). The NO-

adduct also provides effective protection against ulceration of the gut mucosa and may allow these compounds to be better tolerated long-term (Ukawa *et al.*, 1998; Del Soldato *et al.*, 1999; Wallace *et al.*, 1999; Bandarage *et al.*, 2000; Tashima *et al.*, 2000). In particular, S-nitrosated diclofenac analogues should avoid the strict metabolism requirements of the nitrate-NSAIDs and, therefore, might not be expected to induce nitrate tolerance (Bandarage *et al.*, 2000). Further work is now required to establish to what extent NO generation and/or inhibition of eicosanoid synthesis contributes to the therapeutic benefits of these compounds (Lechi *et al.*, 1996; Wallace *et al.*, 1999).

1.8.5.3 Compounds containing a furoxanyl moiety

The dihydropyridine class of calcium antagonists can be linked to the NO-donating furoxans (Di Stilo *et al.*, 1998; Fig 1.16). These compounds cause vasodilatation through sGC stimulation as well as inhibition of voltage-dependent Ca^{2+} ion channels on VSMCs. The same group have also linked furoxans to α_1- (Fruttero *et al.*, 1995) and β_1-antagonists (Boschi *et al.*, 1997). Both produce vasodilatation of isolated aortic strips through a combination of adrenoreceptor antagonism and NO release. Furoxans have also been attached to a histamine H_2 receptor antagonist, and provide a greater protection against gastric ulcers than anti-histamine drugs alone (Coruzzi *et al.*, 2000). These effectiveness of these compounds in pathophysiological models *in vivo* still remains to be tested.

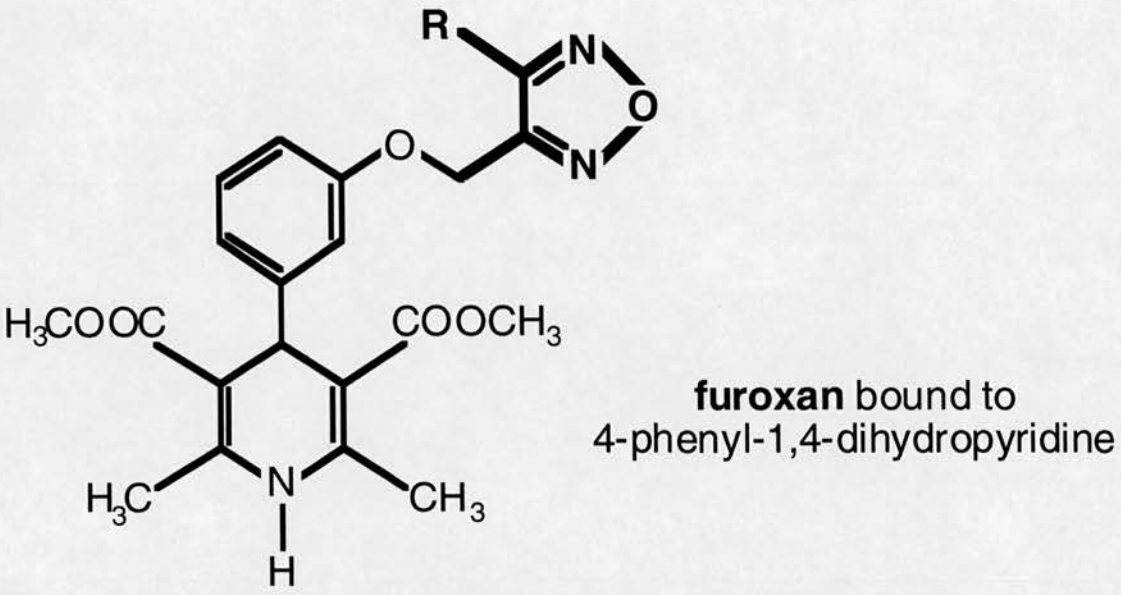
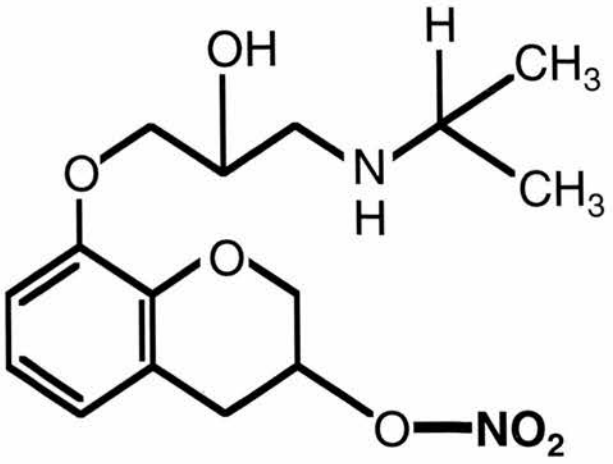


Figure 1.16 Chemical structure of an example of a furoxan-Ca^{2+} channel antagonist hybrid. Area highlighted in bold represents the structural changes allowing addition of a furoxan group to the dihydropyridine group.

1.8.5.4 Nipradilol

It was hypothesised that the combining of a nitrovasodilator and adrenoreceptor antagonist would provide additional beneficial cardiovascular actions by counteracting the undesirable side-effects of the individual drugs. Nipradilol (K-351; Fig 1.17) is an orally active compound that was first described in the early 1980s as a non-specific β-receptor antagonist containing a nitroxy ester group (Uchida *et al.*, 1983). Addition of this group instils the drug with vasodilator actions similar to GTN, as well as enhancing the β-antagonistic potency and providing antagonism for α-receptors (Uchida *et al.*, 1983). There is conflicting evidence as to whether nipradilol shows selectivity for large (Uchida *et al.*, 1983) or small (Lamping & Bloom, 1995) arteries. Recently, Thakur *et al* demonstrated that nipradilol inhibits the development of atherosclerotic lesions in a rabbit model of hypercholesterolaemia and eNOS inhibition, although the efficacy of the parent compound was not tested (Thakur *et al.*, 2002). Interestingly, the beneficial actions of nipradilol appear to be partially due to increased eNOS expression (Jayachandran *et al.*, 2001; Thakur *et al.*, 2002).



Nipradilol

Figure 1.17 Chemical structure of nipradilol.

The binding of nipradilol to β_2-receptors is required for both nipradilol-derived NO release and eNOS potentiation (Jayachandran *et al.*, 2001). The beneficial effects of nipradilol in cardiovascular conditions have been well studied in both animal models and small clinical studies (Hayashi & Iguchi, 1998), although no large-scale clinical trials have been carried out. It appears that nipradilol administered twice daily retains its antihypertensive potency over 24 h (Haneda *et al.*, 1995). However, from a mechanistic point of view, it has yet to be fully established whether continuous use leads to loss of the nitrate-related actions due to the development of tolerance, without necessarily diminishing the beneficial effects mediated through adrenoreceptor antagonism

1.8.6 S-Nitrosothiols

1.8.6.1 Endogenous S-nitrosothiols

S-Nitrosothiols (general formula R-S-N=O) are nitrosated derivatives of the ubiquitous biological molecules, thiols. S-nitrosothiols are found endogenously and include S-

nitrosoalbumin (SNO-Alb), the S-nitrosated form of the most abundant thiol in blood plasma (Stamler *et al.*, 1992; Marley *et al.*, 2001), S-nitrosoglutathione (GSNO; Fig 1.18a), the S-nitrosated form of the most common intracellular thiol (Gaston *et al.*, 1993), SNOC (Fig 1.18b), an S-nitrosated low molecule weight thiol perhaps released from endothelial cells (Myers *et al.*, 1990) and S-nitrosohomocysteine, which generates less reactive oxygen species than its parent thiol, homocysteine (Stamler *et al.*, 1993). S-Nitrosothiol complexes stabilise NO and probably represent an endogenous reservoir and/or intra/inter-cellular carriers of NO.

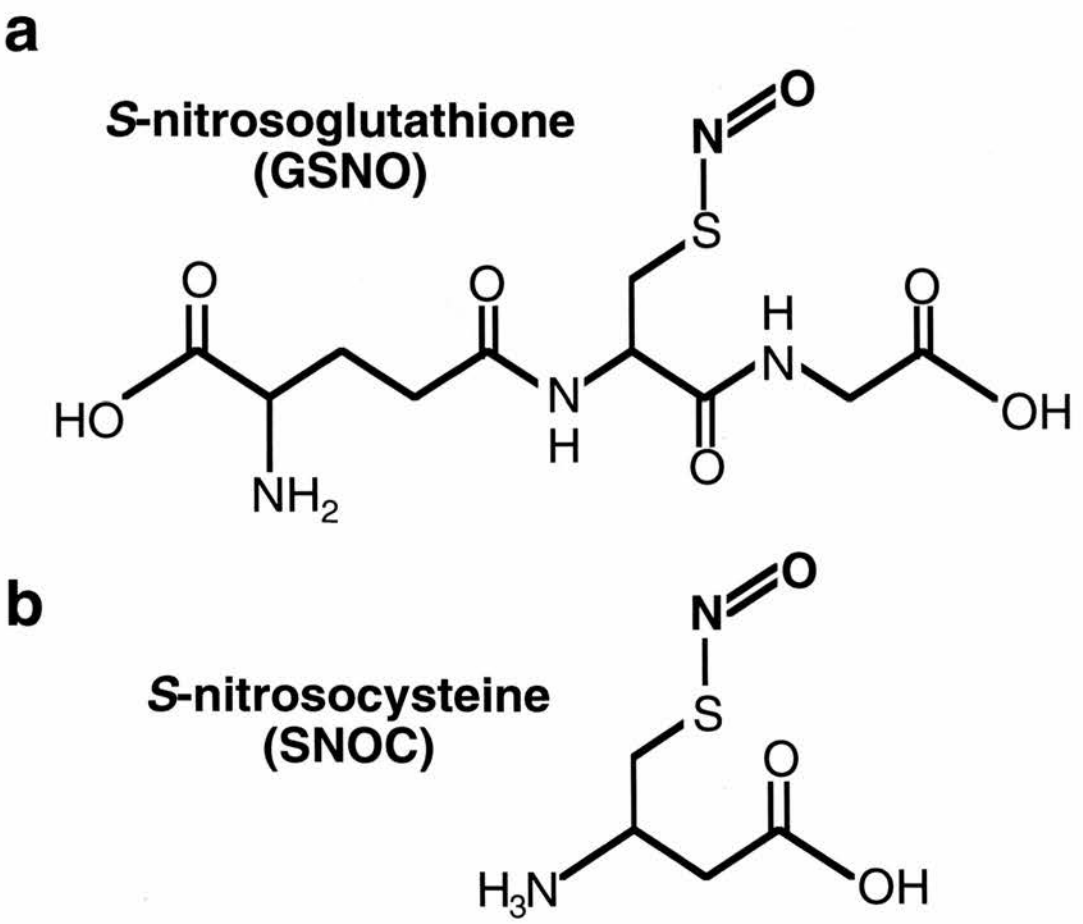


Figure 1.18 Chemical structure of two endogenous S-nitrosothiols; (a) GSNO, (b) SNOC.

As well as binding to the haem group of red blood cell Hb, the thiol groups of Hb can be S-nitrosated, forming S-nitrosohaemoglobin (Jia *et al.*, 1996). This is a particularly

controversial area of NO biology and there is increasing evidence that both oxygen and NO are carried by haemoglobin in red blood cells, allosterically regulating the affinity for each other to provide a dynamic control of oxygen delivery and blood vessel tone (Jia *et al.*, 1996; Wolzt *et al.*, 1999; Gladwin *et al.*, 2000).

1.8.6.2 Mechanisms of action

Classically, the bioactivity of S-nitrosothiols requires decomposition to free NO. At one time, the *in vitro* stability of S-nitrosothiols was unpredictable. This was due to the presence of trace metal ions in experimental solutions. The use of specific copper (I) chelators demonstrated that Cu(I) ions catalyse the decomposition of S-nitrosothiols (Dicks *et al.*, 1996; Al-Sa'doni *et al.*, 1997; Butler *et al.*, 1998) producing NO and the corresponding disulphide (Fig 1.19). The stability of S-nitrosothiols depends upon the structure of the thiol which can inhibit Cu-catalysed decomposition by steric hindrance of the S-N bond (Mathews & Kerr, 1993). Free thiols, and other reducing agents, will accelerate the decomposition of S-nitrosothiols by reducing Cu(II) to Cu(I) ions (Dicks *et al.*, 1996; Singh *et al.*, 1996; Al-Sa'doni *et al.*, 1997; Holmes & Williams, 1998).

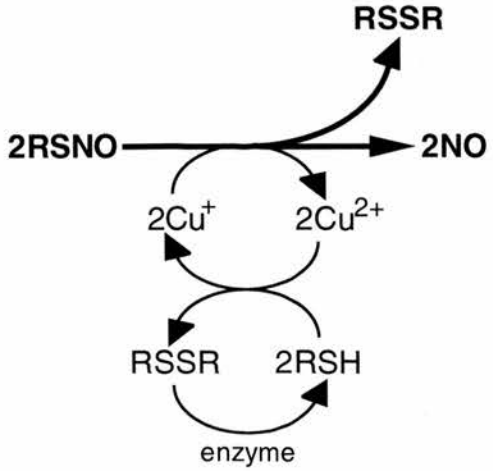


Figure 1.19 Pathway of Cu-catalysed NO release from nitrosothiols and recycling and thiols and copper ions.

Superoxide generation has also been shown to accelerate the decomposition of S-nitrosothiols (Aleryani *et al.*, 1998; Jourd'heiul *et al.*, 1998; Trujillo *et al.*, 1998). The biological actions of S-nitrosothiols such as SNOC (Davisson *et al.*, 1996) or S-nitrosopenicillamine (Travis *et al.*, 1996; Travis *et al.*, 1997) show stereoselectivity, suggesting the involvement of enzymes. A number of enzymes have been implicated in the decomposition of S-nitrosothiols including SOD (Jourd'heuil *et al.*, 1999), glutathione peroxidase (Freedman *et al.*, 1995; Hou *et al.*, 1996), xanthine oxidase (Trujillo *et al.*, 1998), protein dulpshide isomerase (PDI; Zai *et al.*, 1999; Ramachandran *et al.*, 2001) and an unidentified membrane bound protein in vascular smooth muscle cells (Kowaluk & Fung, 1990). Despite variable half-lives, different S-nitrosothiols have similar bioactivity, suggesting a common mechanism of action (Kowaluk & Fung, 1990; Mathews & Kerr, 1993).

A unique feature of S-nitrosothiols is that they can directly transfer NO as a nitrosonium ion (NO^+) without the release of free NO ("transnitrosation"; Park, 1988; Askew *et al.*, 1995; Eqn 10).

$$R^1SNO + R^2SH \rightarrow R^1SH + R^2SNO \quad (10)$$

Therefore, free thiols also accelerate the decomposition of S-nitrosothiols by providing an acceptor for NO^+. Transnitrosation of a thiol group could form a S-nitrosothiol more susceptible to Cu-mediated decomposition (e.g. GSNO->SNOC), leading to the release of free NO in specific subcellular sites (Park, 1988; Askew *et al.*, 1995; Liu *et al.*, 1998). S-nitrosation of SH residues in enzymes and proteins modifies their activity, providing another level of regulatory activity (Stamler *et al.*, 1992; Lipton *et al.*, 1993; Molina *et al.*, 1993; Bolotina *et al.*, 1994; Melino *et al.*, 1997; Clementi *et al.*, 1998; Bauer *et al.*, 1999; Xie *et al.*, 1999).

All S-nitrosothiols are potent inhibitors of platelet aggregation *in vitro* (Gordge *et al.*, 1998; Sogo *et al.*, 2000) and inhibit platelet activation *in vivo* (Radomski *et al.*, 1992) at concentrations which do not effect blood vessel tone (De Belder *et al.*, 1994). S-nitrosothiols also show arterioselectivity, inhibiting vasodilatation at a concentration

which causes little venodilatation (MacAllister *et al.*, 1995). Because S-nitrosothiols have a less stringent biotransformation pathway than organic nitrates, it is unsurprising that S-nitrosothiols do not develop tolerance and remain effective in GTN-tolerant vessels *in vitro* (Kowaluk *et al.*, 1987; Kowaluk & Fung, 1990; Matsumoto *et al.*, 1995) and *in vivo* (Bauer & Fung, 1991; Shaffer *et al.*, 1992).

1.8.6.3 Therapeutic applications

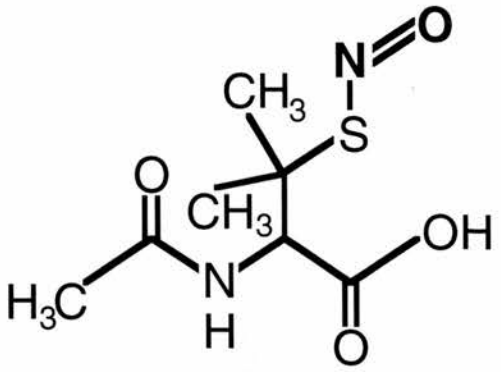
There is a large body of literature exploring the use S-nitrosothiols in animal models and clinical studies. In general, most studies focus on the use of GSNO because of its relative stability and likelihood that it generates non-toxic, or even beneficial, by-products (i.e. the antioxidant GSH or its disulphide, GSSG). The anti-platelet actions of GSNO have beneficial actions in angina and myocardial infarction in patients already receiving aspirin (Langford *et al.*, 1996). In this study, GSNO was better tolerated than GTN. GSNO also decreases the occurrence of cerebral embolism after carotid endarterectomy in patients already receiving aspirin and heparin (Molloy *et al.*, 1998). GSNO prevents platelet activation following balloon angioplasty (Langford *et al.*, 1994), whilst SNO-Alb, administered as an infusion (Marks *et al.*, 1995) or as a stent-coating (Maalej *et al.*, 1999), has been shown to reduce platelet adhesion and neointimal thickening in balloon angioplasty-damaged blood vessels. GSNO also reduces platelet adhesion in bypass grafts (Salas *et al.*, 1998).

At present it is not clear whether S-nitrosothiols can be given orally, but transdermal delivery of the S-nitrosothiol or (S-nitrosothiol-derived NO) is feasible and may have a therapeutic role in Raynaud's syndrome and impotence (Butler *et al.*, 1997; Khan *et al.*, 1997).

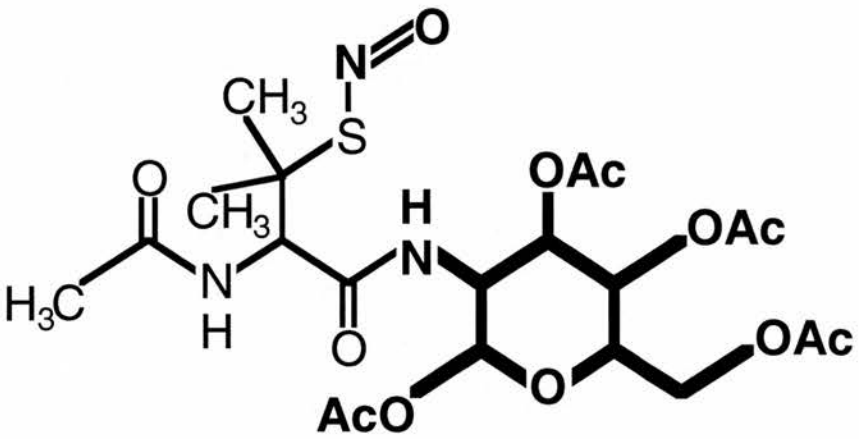
1.8.6.4 Novel S-nitrosothiols

Recently, several novel analogues of the S-nitrosothiol S-nitroso-*N*-acetylpenicillamine (SNAP) were described. Structural modifications were made to the thiol group to increase stability by creating steric hinderance of the S-N bond. However, an additional property was discovered for one the compounds, N-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-β-D-glucopyranose (RIG200; Fig 1.20). RIG200 is essentially SNAP with a acetylated glycosamine group (Megson *et al.*, 1997). In perfused isolated vessels, a bolus of RIG200 into the vessel lumen of endothelium-intact arteries produced a transient vasodilatation that recovered rapidly after the compound washed out of the vessel. However, in endothelium-denuded vessels, an identical bolus produced a vasodilatation which did not recover rapidly, but was sustained for a number of hours (Fig 1.21). The sustained vasodilatation could be reversed by the NO scavenger, Hb, but not the NOS inhibitor L-NAME (Megson *et al.*, 1997), showing that the effect was mediated by NO which was not derived from NOS. The parent compound, SNAP, did not produce a sustained vasodilatation in denuded arteries.

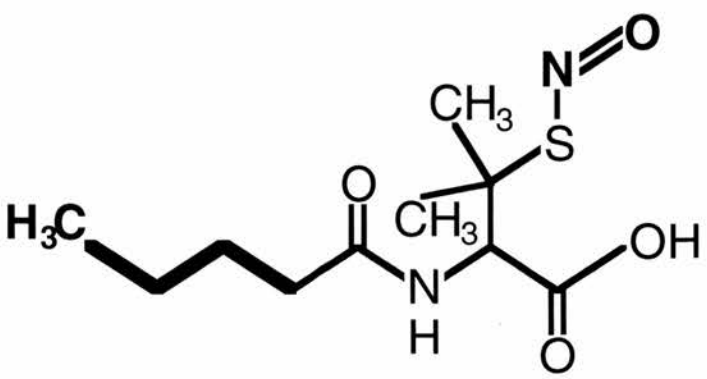
Other analogues of SNAP were synthesised containing N-substituted carbon side-chains, which, as well as increasing stability, also had the effect of altering the lipophilicity of the compound (Megson *et al.*, 1999). It was shown that the lipophilic compounds, such as S-nitroso-*N*-valeryl-penicillamine (SNVP; five carbon side-chain; Fig 1.20) also produced a sustained vasodilatation in endothelium-denuded arteries. Moreover, there was a strong correlation between the lipophilicity and the lack of recovery of vasodilatation after 1 h (Fig 1.21). Again, the effect was reversed by Hb. The authors hypothesised that the endothelium acts as a barrier to these compounds and that, in endothelium-denuded arteries, the lipophilic S-nitrosothiols gain access to the lipid-rich sub-endothelial layer, where they are retained. Here, they slowly decompose to NO producing a sustained vasodilatation (Megson *et al.*, 1999; Fig 1.22).



S-nitroso-N-acetylpenicillamine (SNAP)



N-(S-nitroso-N-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-O-acetyl-ß-D-glucopyrnaose (RIG200)



S-nitroso-N-valerylpenicillamine (SNVP)

Figure 1.20 Chemical structures of SNAP, RIG200 and SNVP. Areas highlighted in bold represent the structural changes to SNAP, engendering the compound with additional stability and lipophilicity.

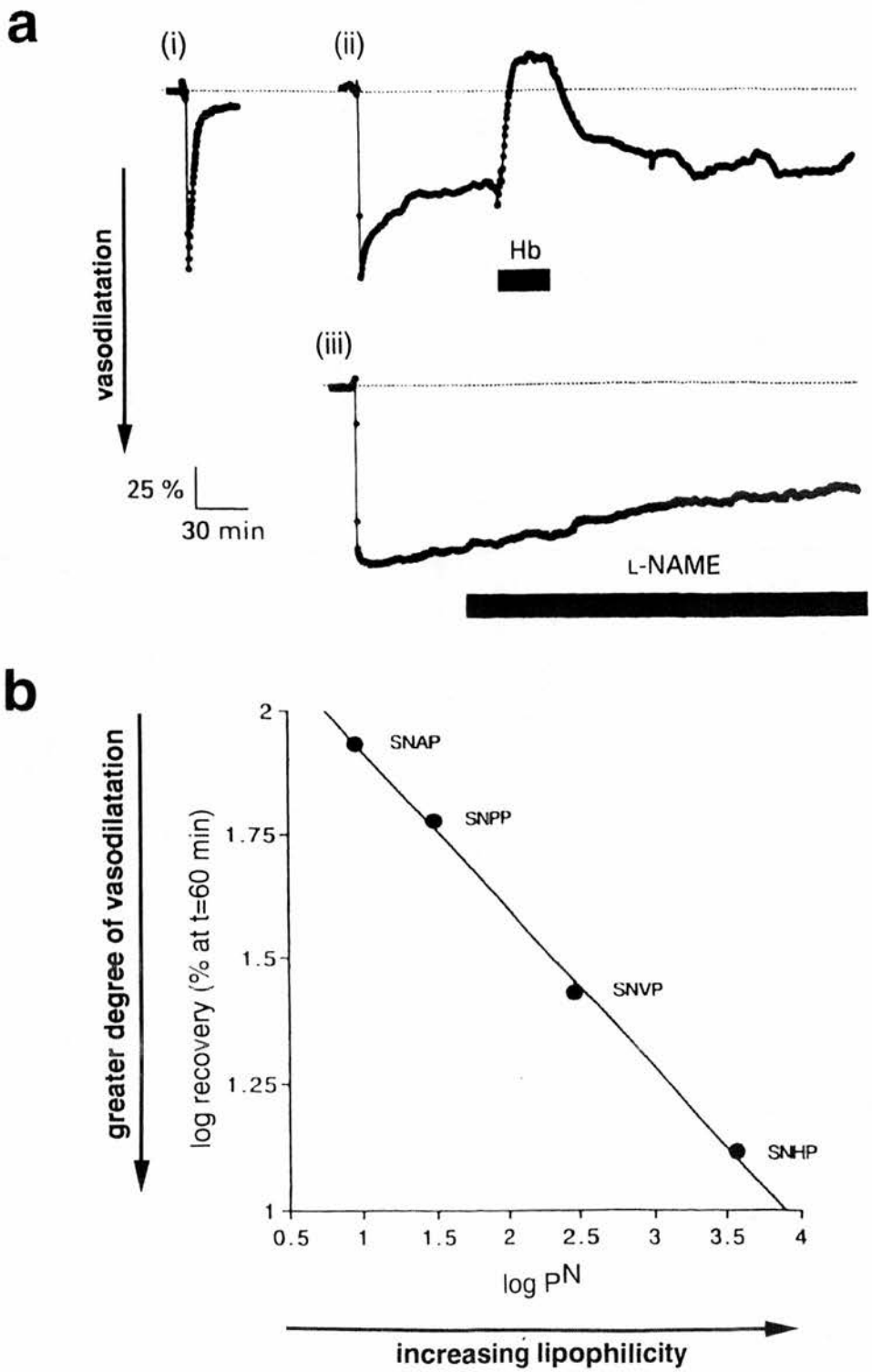


Figure 1.21 Results from experiments on phenylephrine-contracted isolated rat femoral arteries. (a) In endothelium intact vessels (i), a bolus of RIG200 (10 µl; 10^{-3} M) produces a transient vasodilatation which recovers rapidly after the bolus washes through the vessel lumen. However, in endothelial-denuded vessels (ii, iii), an identical bolus of RIG200 produces a vasodilatation which is sustained for a number of hours. This vasodilatation can be reversed by the NO scavenger, Hb (10 µM; ii), but not by the NOS inhibitor, L-NAME (200 µM; iii). Taken from Megson *et al.* (1997). Br. J. Pharmacol. **122**, 1617-1624. (b) Data from boluses of SNAP analogues demonstrating that the greater the lipophilicity of the S-nitrosothiol, the greater the degree of vasodilatation that remains after 1 h. Taken from from Megson *et al.* (1999). Br. J. Pharmacol. **126**, 639-648.

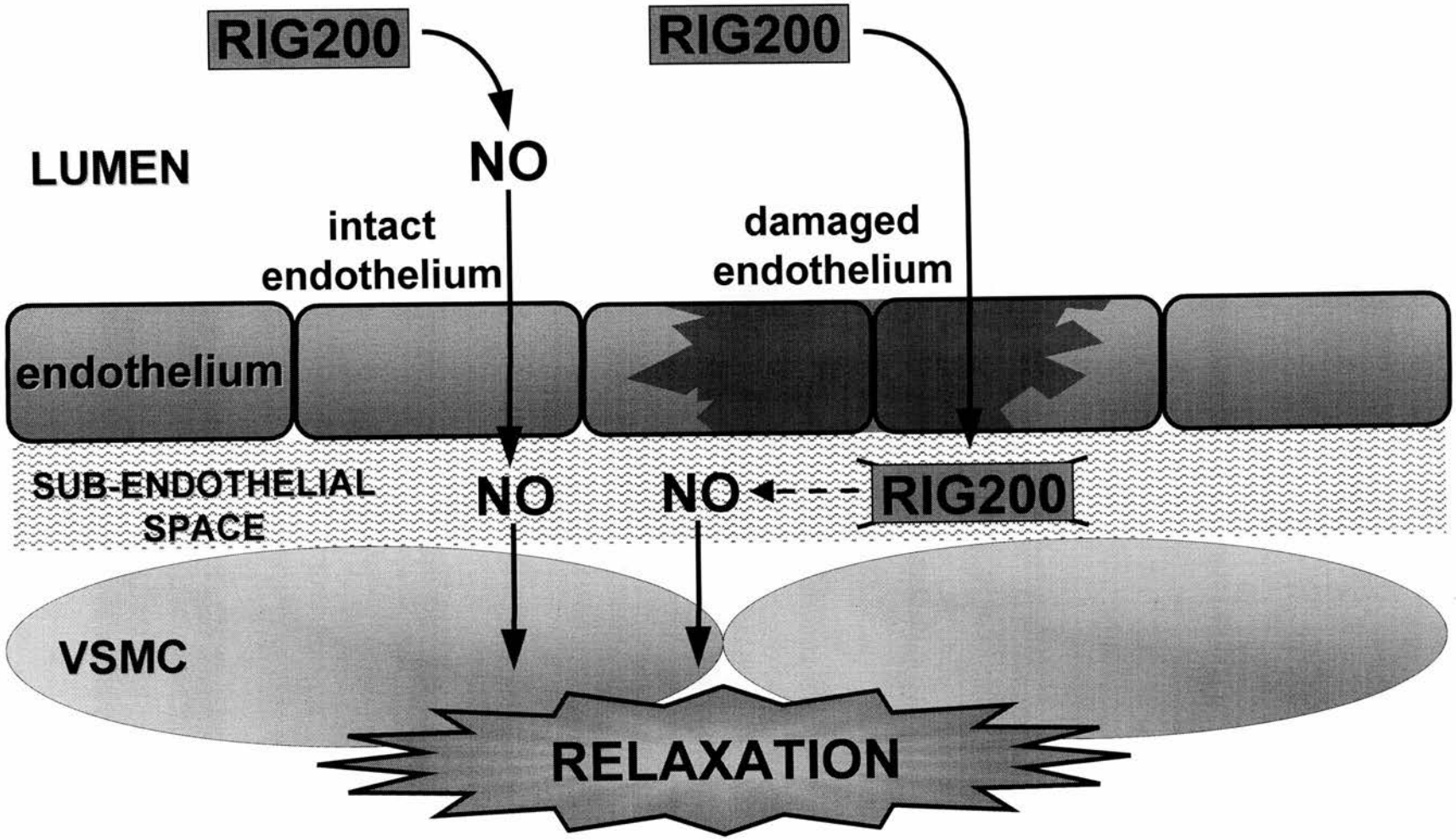


Figure 1.22 Schematic diagram showing the hypothesis of sustained vasodilatation caused by lipophilic S-nitrosothiols. The endothelium acts as a barrier to S-nitrosothiols, which produce a transient vasodilatation through decomposition to NO. At areas of endotheial damage, lipophilic S-nitrosothiols are retained in the lipid-rich areas of the subendothelial space. Here they slowly decompose to NO producing a sustained vasodilatation.

More recently, it has been shown that these lipophilic S-nitrosothiols produce a sustained vasodilatation in human blood vessels *in vitro* (Sogo *et al.*, 2000) and *in vivo* (Sogo *et al.*, 2000). Additionally, these compounds, like conventional S-nitrosothiols, are potent anti-platelet agents in platelet-rich plasma (Sogo *et al.*, 2000), but not in mixed whole blood (Megson *et al.*, 2000). This observation suggests that these compounds can inhibit platelet activation in the red-blood cell free layer adjacent to the endothelium in flowing blood *in vivo*, without extending bleeding time at sites of injury where the red blood cells mix with platelets, inhibiting S-nitrosothiol activity (Megson *et al.*, 2000).

1.9 PROJECT AIMS

Despite the clear advantages of S-nitrosothiols, their mechanism of action is still poorly understood. In particular, it has yet to be established if novel S-nitrosothiols behave in a similar fashion to existing S-nitrosothiols, or whether their structural adaptations alter their vasodilatation in additional ways, such as their ability to induce sGC-independent vasodilatation or their susceptibility to vascular tolerance.

The sustained NO-mediated vasodilator effect of lipophilic S-nitrosothiols in endothelium-denuded vessels is a particularly attractive feature. However, it remains to be established whether the prolonged NO-mediated effects of these compounds has other biologically relevant actions, such as inhibition of platelet activation. Furthermore, it is as yet unclear whether the potentially beneficial properties of these compounds are mirrored in therapeutically relevant applications, such as balloon angioplasty.

This thesis focuses on cardiovascular effects of novel lipophilic S-nitrosothiols. The thesis can essentially be split into two sections. The first section focuses on the action of S-nitrosothiols in endothelium-intact blood vessels. Initially, isolated vessels are used to clarify the mechanism of action of these novel compounds, by comparison to established NO donor drugs. Secondly, the thesis deals with the effects of novel lipophilic S-nitrosothiols in vessels with a damaged endothelium. Both the mechanism of the sustained actions in denuded vessels *in vitro* and the therapeutic potential *in vivo* are considered. It is hypothesised that;

- S-nitrosothiols cause vasodilatation partially through sGC-independent mechanisms.

- sGC-independent vasodilatation is not mediated by peroxynitrite formation.

- cell surface thiols are required for the vasodilatory actions of S-nitrosothiols.

- intracellular thiols are required for the vasodilatory actions of all NO donors.

- lipophilic S-nitrosothiols retain the tolerance-profile of their parent compound, i.e. they do not induce self-tolerance and remain fully active in vessels made tolerant to GTN.

- a hydrophilic analogue of RIG200 does not cause a sustained vasodilatation in isolated endothelium-denuded vessels.

- SNVP selectively causes vasodilatation and reduces platelet adhesion to blood vessels denuded of endothelium following balloon angioplasty in vivo.

Chapter 2

Methods

2. METHODS

2.1 DETECTION OF S-NITROSOTHIOLS USING SPECTRO-PHOTOMETRY

S-nitrosothiols (1 ml; 1 mM) were dissolved in identical buffer used for experiments with biological tissue. Sonication (35 μm, 5-10 s, x3; Soniprep 150 sonicator, Sanyo, Uxbridge, U.K.) was needed to dissolve some S-nitrosothiols (GSNO, RIG200, SNVP). Samples were kept in the dark at 24°C. Peak absorbance at a wavelength corresponding to the S-N bond (330-340 nm) was measured using a microspectrophotometer (Spectromate, World Precision Instruments, Stevenage, U.K.). Extinction coefficients (850-1168 $M^{-1}cm^{-1}$) were compared to literature values (Gordge *et al.*, 1996; Megson *et al.*, 1997; Megson *et al.*, 1999) to ensure full dissolution and lack of spontaneous decomposition.

2.2 MEASUREMENT OF NO USING NO ELECTRODE

Two ml samples of Krebs buffer solution were prewarmed to 37°C in cuvettes stirred continuously at 1000 rpm. An isolated NO electrode (ISO-NO MARKII, World Precision Instruments, Stevenage, UK) was introduced into the cuvette and allowed to stabilise (10-30 min). Once a stable baseline was obtained, the electrode was calibrated using 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO; 100-800 nM) in phosphate buffer (pH 4). DEA/NO undergoes rapid, spontaneous decomposition at pH<4 (Davies *et al.*, 2001). Krebs buffer (pH 7.4) was used for subsequent

protocols. NO donors were introduced into the cuvette and NO generation was allowed to reach plateau, prior to further addition of drugs. In experiments to determine the role of tissue components in superoxide generation, 1 cm segments of rat aorta were homogenised in Krebs buffer (50 µl) using a micropestle (Eppendorf, Cambridge, U.K.). Supernatant (40 µl) was removed and added to cuvettes, before addition of NO donors. Pilot experiments determined the limits for detection of NO in this system as ~20 nM. Signals from the NO electrode were processed by a MacLab/4e analogue-digital converter and displayed through Chart™ software (AD Instruments, Sussex, U.K.) on a Macintosh Performa 630 microcomputer.

2.3 FUNCTIONAL STUDIES USING PERFUSED ISOLATED RAT FEMORAL ARTERIES

All experiments using animals were carried out in accordance with the Animals (Scientific Procedures) Act 1986 (U.K. Home Office).

2.3.1 Preparation of femoral arteries

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (250-350 g) in a perfusion system described previously (Megson *et al.*, 1997; Fig 2.1). Briefly, animals were killed by cervical dislocation and both femoral arteries were dissected free. Segments of artery (7-8 mm long) were cannulated immediately distal to the epigastric arterial branch. The vessels were transferred to perspex organ chambers (1 ml volume) where they were perfused (0.6 ml min^{-1}: Gilson Miniplus 3; Anachem, Luton, U.K.) and superfused (1 ml min^{-1}: Watson Marlow 302S; Falmouth, U.K.) with fresh oxygenated (95% O_2, 5% CO_2) Krebs buffer (see Appendix I for composition) at 37°C. The contractile state of the vessel

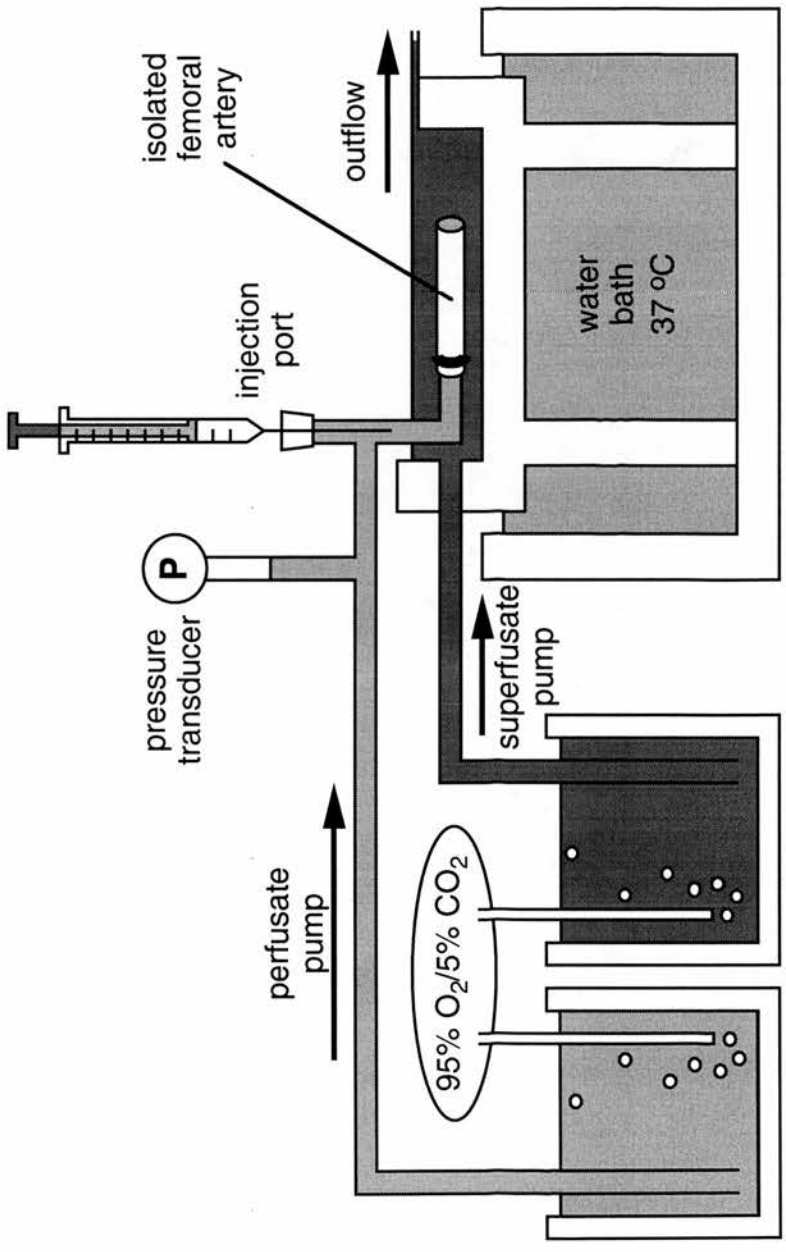


Figure 2.1 Schematic diagram of the perfusion system used to investigate vasodilator responses in rat isolated femoral arteries.

was measured by monitoring perfusion pressure with a differential pressure transducer (Sensym SCX 15ANC; Farnell Electronic Components, Leeds, U.K.) located upstream of the artery. The apparatus permits exclusive drug delivery to the luminal surface of the vessel, either in the perfusate or by bolus microinjection (10 μl) through a resealable rubber septum into the perfusate immediately upstream of the vessel (transit time to artery ~3 s, through lumen ~300 ms). All experiments were carried out in a darkened laboratory in order to protect photolabile drugs, prevent photorelaxation of vessels (Megson *et al.*, 1995) and to minimise spontaneous superoxide generation in oxygenated Krebs buffer (Beckman & Koppenol, 1996). Signals from pressure transducers were processed by a MacLab/4e analogue-digital converter and displayed through Chart™ software.

2.3.2 General experimental protocol

Vessels were preconstricted with phenylephrine (PE; 2-14 μM) in the presence of supramaximal concentrations of the NO synthase inhibitor, L-NAME (20 μM), to exclude endothelial NO-synthase activation in vasodilator responses. The concentration of L-NAME was minimised to prevent toxicity in prolonged (>20 h) perfusion. This concentration of L-NAME was shown to be supramaximal as Hb caused no additional vasoconstrictor effect (see Chapter 3). Perfusion pressure remained constant after prolonged perfusion of L-NAME (Chapter 4 and 5), indicating that iNOS expression was not sufficiently high to overcome the inhibitory effects of this concentration of L-NAME. An L-NAME-induced increase in pressure of >40% of the existing PE-induced tone was deemed to beindicative of an active endothelium. Unless stated, arteries were not denuded of endothelium in order to preserve the potential endothelium-dependent superoxide generating systems (Holland *et al.*, 1990; Munzel *et al.*, 1995), except that generated by NO synthase which may be inhibted by L-NAME (Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998; Munzel *et al.*, 2000).

Concentration response curves were carried out by sequential microinjections of increasing concentrations (10 µl of 10^{-8} - 10^{-3} M; 0.1 pmol - 10 nmol) of NO donors. Alternatively, prolonged administration of compounds could be achieved by adding the compound to the perfusate or superfusate. At the end of experiments, all drugs were washed out and the vessel removed to ensure pressure returned to baseline. Microinjections of drug vehicle (Krebs buffer or saline) had no effect on perfusion pressure. Sequential boluses of NO donors (10 µl; 10^{-8} - 10^{-3} M) had no effect on the magnitude of subsequent microinjections. Peak vasodilatation correlates with the time course of vasodilatation (area under curve; AUC; %preconstriction.sec) in both control vessels and vessels treated with modulators (r=-0.76; $P<0.001$; Pearson's correlation; n=32; Fig 2.2).

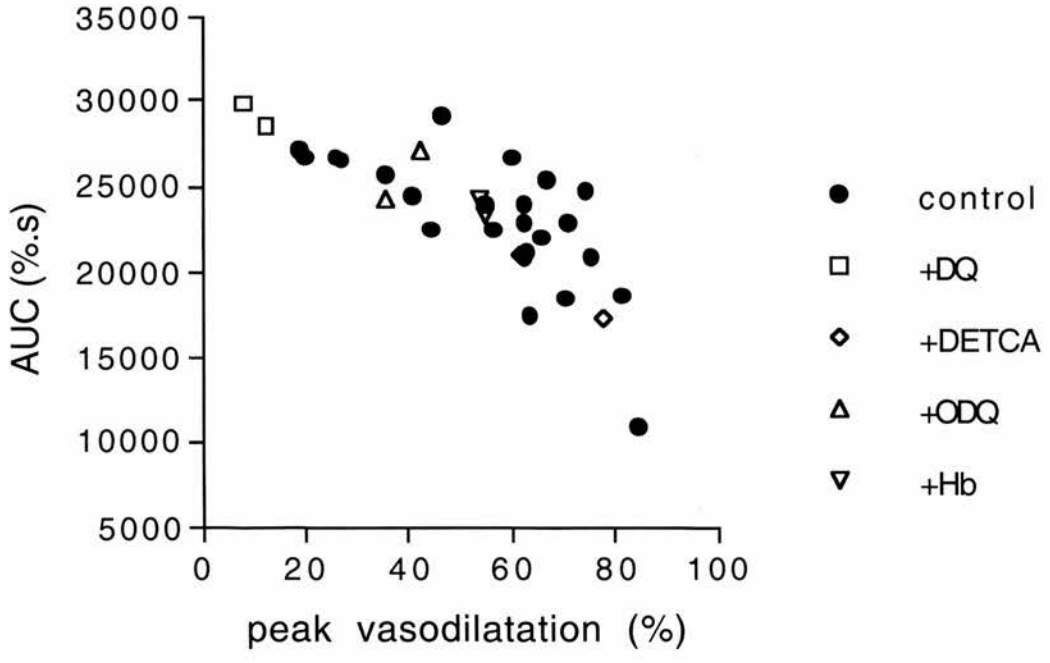


Figure 2.2 Correlation between peak vasodilatation and time course of vasodilatation (both expressed as % preconstriction pressure existing before drug bolus) in control vessels and vessels treated with modulators (r=-0.76; $P<0.001$; Pearson's correlation; n=32).

2.4 ANGIOPLASTY OF RABBIT CAROTID ARTERIES

Adult male New Zealand white rabbits (2.5-3.5 kg; n=60) had unrestricted access to water and standard chow. Animals were anaesthetised with an intramuscular injection of Hypnorm (0.3 ml/kg; fentanyl citrate & fluanisine) and anaesthesia was maintained with a halothane (1.5%) / nitrous oxide (2%) / oxygen (5 $L.min^{-1}$) mixture (Hadoke *et al.*, 1995). The left femoral artery was exposed and a 3FG cannula (Portex Ltd., distributed by Mackay & Lynn Ltd, Edinburgh, U.K.) filled with heparinised-saline (Hep-Sal; 25 U/ml; Multiparin) was advanced ~15 mm into the artery for the withdrawal of blood and the measurement of systemic blood pressure (BP). Five ml of blood was withdrawn into a Monovette™ tube containing 0.4 ml sodium citrate (3.8%) and gently mixed. BP was measured using a pressure transducer (CAPTO SP844 physiological pressure transducer, ADInstruments Ltd, East Sussex, U.K.), displayed through Chart™ software. The external carotid artery was cannulated and a 2.5x20 mm angioplasty catheter (Boston Scientific SCIMED, Galway, Ireland) was introduced into the common carotid artery (Fig 2.3). Angioplasty was performed on a 40 mm section of artery, using 30 s inflations (10 atmospheres of pressure; atm; 2 times with a 15 s interval). The balloon was withdrawn under 4 atm. Sham operations involved cannulation of the common carotid artery without balloon inflation. The artery was recannulated to administer drug boluses (200 nmoles; flushed through the cannula with a further 0.2 ml of Hep-Sal) immediately upstream of the angioplastied region. Blood was allowed to flow over the angioplastied region for 30 min after drug administration (~35 minutes after angioplasty). Animals were killed with an overdose of pentobarbitone and both common carotid arteries were dissected free and placed in Krebs buffer. Arteries were cleaned of connective tissue and divided into rings for further assessment. Vessel length was measured using calipers.

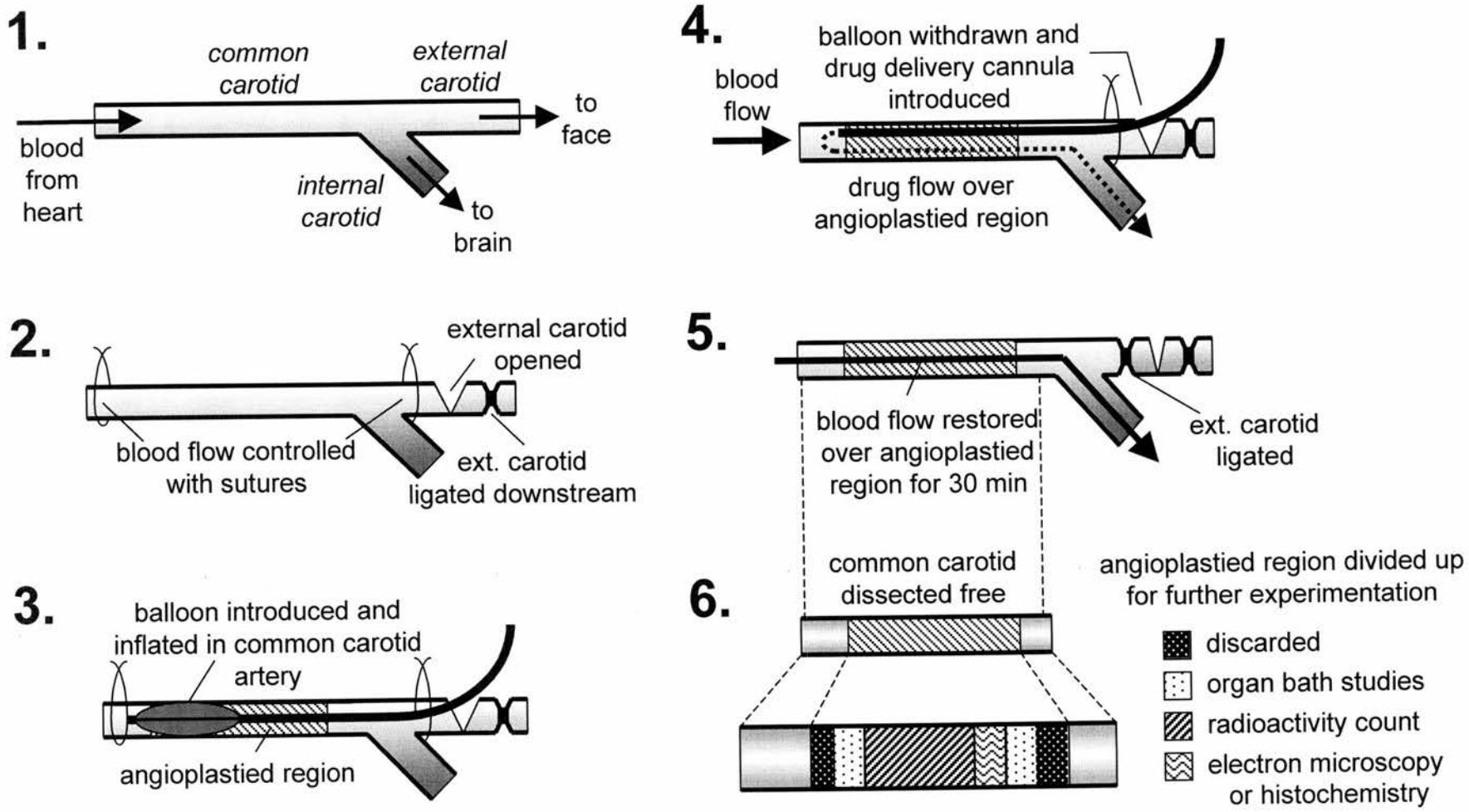


Figure 2.3 Schematic diagram of the treatment and utilisation of the common carotid artery in angioplasty experiments. The hatched area represents the angioplastied region, which is isolated and divided up, as indicated, for further experimentation.

2.5 FUNCTIONAL STUDIES USING ISOLATED RABBIT CAROTID ARTERIES

2.5.1 Preparation of carotid rings

Rabbit common carotid arteries were isolated, cleaned of connective tissue and divided into rings (3mm). Vessel rings were suspended between two intraluminal wires in a 10 ml myograph organ bath (Multi Tissue Bath System 700MO, Danish Myo Technology A/S, Copenhagen, Denmark) to record isometric tension. Vessels were bathed in oxygenated (95% O_2, 5% CO_2) Krebs solution at 37°C. Tension was applied to vessels in stepwise increments to obtain a resting tension of 7 g (Dong *et al.*, 1997) and allowed to equilibrate for 30-40 min. Signals from the myograph were processed by a MacLab/4e analogue-digital converter and displayed through Chart™ software.

2.5.2 General experimental protocol

Rings were contracted three times to obtain the maximum contraction to high K^+ Krebs, containing equimolar replacement of NaCl (4.7 mM) with KCl (118 mM; see Appendix I). Rings were subsequently exposed to cumulative concentrations of PE (0.1-10 μM) and a suitable concentration chosen to produce ~80% contraction (EC_{80}; ~3 μM). Following precontraction with EC_{80} PE, responses to ACh (0.01-30 μM) were measured to test endothelial cell function.

2.6 ISOLATION AND RADIOLABELLING OF PLATELETS

Citrated blood was centrifuged at 200 *g* for 10 min. Platelet-rich plasma (PRP) was aspirated and centrifuged at 1300 *g* for 10 min with prostacyclin (PGI_2; 300 ng/ml) to pellet platelets. Platelet poor plasma was removed and the pelleted platelets were resuspended and incubated for 20 min in 10 ml Tyrode's solution containing 100 $\mu Ci^{111}InCl_3$ chelated to 2-mercaptopyridine-N-oxide (MERC; 400 µg/ml). Platelets were washed three times by centrifugation at 1300 *g* for 10 min and resuspended in PGI_2/Tyrode's solution to remove unbound radiolabel. Finally, platelets were resuspended in PGI_2-free Tyrode's containing ~330 $x10^6$ platelets/ml (determined with a Coulter A^C.T 8 Haematology Analyser, Coulter Electronics Ltd, Luton, U.K.). After each wash cycle, 10 µl samples were taken to determine the labelling efficiency. Approximately 1 ml platelet suspension (50-300 $x10^6$ platelets; radioactivity=50-800 $x10^3$ dpm) was obtained.

Radioactive blood samples (100 µl) and segments of carotid artery (~5 mm) were added to 4 ml scintillation fluid. Radioactivity was assessed by a Tri-Carb™ 1900TR Liquid Scintillation Analyser (Packard Instrument Company, Meriden, U.S.A.)

2.7 PLATELET AGGREGATION IN RESPONSE TO AGONISTS

Half-milliliter samples of PRP were pre-warmed to 37°C for 5 min in a two-channel platelet aggregometer (Cronolog Ca560, Labmedics, Stockport, U.K.) capable of measuring aggregation in PRP by turbidometry by a standard method (Megson *et al.*, 2000).

Samples of PRP were stirred at 1000 r.p.m. using disposable stirrer bars and platelets were activated with supramaximal concentrations of adenosine 5'-diphosphate

(ADP; 8 μM). Aggregation was measured by monitoring changes in light transmission for a period of 5 min following agonist addition.

2.8 MEASUREMENT OF PLASMA CATECHOLAMINES

High performance liquid chromatography (HPLC) was used to measure plasma adrenaline, noradrenaline and dopamine. This work was carried out by Dr R. Stephen, Department of Child Life and Health, University of Edinburgh, by a published method (Sedowofia *et al.*, 1998). Briefly, PRP (0.5 ml) was frozen immediately after centrifugation (Section 2.6). Plasma catecholamines were measured by dual-electrode coulometric detection (ESA Coulochem Model 5100A) after separation on a reverse phase HPLC column (Waters 510 pump with pulse dampener, equipped with a Promis 11 programmable auto-injection system; Spark Holland BU), using a modification of the method of Davies *et al.* (Davies & Molyneux, 1982). The upstream and downstream electrodes were set at +0.25 V and -0.4 V, respectively. A simple solvent extraction system was used for the selective and quantitative isolation of catecholamines, using a modification of the method of Smedes *et al.* (Smedes *et al.*, 1982). The limits of detection were <5 pg per injection for adrenaline and noradrenaline and <10 pg per injection for dopamine. The interassay coefficients of variation for adrenaline, noradrenaline and dopamine were 2.0%, 1.6% and 3.4%, respectively. Dihydroxybenzylamine was used as the internal standard.

2.9 VISUALISATION OF BLOOD VESSELS USING ELECTRON MICROSCOPY

Blood vessels (3 mm) were carefully cut along the longitudinal axis to expose the vessel lumen and pinned out on an agar bed. The vessel was then immersed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB; pH 7.4; see Appendix I) for 8-10 h, followed by 1 h glutaraldehyde-free SCB wash. Vessels were postfixed in osmium tetroxide in SCB (1-2 h), dehydrated in graded acetone (50-100%; 10 min intervals) and critical point dried with CO_2 (E3000 SII CPD, Polaron Equipment Ltd, Watford, U.K.). The vessel was coated with gold-palladium alloy (SC500 Sputter coater, Emscope Laboratories Ltd, Kent, U.K.) and the intimal surface was examined with a Phillips 505 scanning electron microscope (Eindhoven, Netherlands). Representative surfaces were photographed.

Following fixation and dehydration, vessel rings were imbedded in 50:50 araldite:acetone. 60nm sections were cut (Reichert OMU4 Ultracut microtome, Leica, Milton Keynes, U.K.) and mounted on 200 mesh copper grids. Uranyl acetate and lead citrate were used to stain sections (LKB Ultrostainer, LKB, Surrey, U.K.) as per manufacturer's instructions. Samples were examined with a Phillips CM12 transmission electron microscope (Eindhoven, Netherlands). Representative sections were photographed.

2.10 HISTOCHEMICAL ANALYSIS OF BLOOD VESSEL STRUCTURE

2.10.1 Preparation of sections

Blood vessels (3mm) were fixed in formalin (10%) for 24 hr and then placed in 70% alcohol. Samples were embedded in parafin wax blocks and 3 μm cross-sections were cut (Leitz 1512 microtome, Carl Zeiss, Hertfordshire, U.K.). Sections were floated out and placed on 3-aminopropyltriethoxy-saline (TESPA)-coated slides (see Appendix I) and allowed to adhere overnight at 37°C. Sections were dewaxed in 100%

xylene (2x5 min) and then rehydrated in in graded alcohol (100-70%, distilled water; 2-5 min intervals). Sections were then washed in phosphate buffered saline (PBS; pH 7.6; 3x3 min; see Appendix I). Following staining, sections were dehydrated in graded alcohol (distilled water, 70-100%; 2 min intervals) and placed in xylene (2x5 min). Coverslips were attached with DePeX .

All washes were in PBS or Tris-buffered saline (TBS; pH 7.6; see Appendix I) for 3x3 min. Serum and antibodies were diluted in 0.5% bovine serum albumin (BSA).

2.10.2 Haematoxylin and eosin staining

Rehydrated sections were placed in Harris' haematoxylin for 10 min and then washed in running water until the water ran clear (~2 min). Sections were placed in 1% acetic acid (diluted in 70% alcohol) for 30s and then washed in running water for 5 min. Sections were counterstained with eosin (1%) for 2 min, washed in distilled water, dehydrated and coverslips affixed.

2.10.3 Immunohistochemistry

Rehydrated sections were warmed in TBS at 37°C for 10 min, followed by incubation with trypsin-TBS (see Appendix I) at 37°C for 45 min and a PBS wash. Sections were covered 50:50 rabbit serum:BSA-PBS (see Appendix I) for 30 min. Excess serum was removed and the primary antibody (unconjugated Griffonia (Bandeiraea) Simplicifolia Lectin I; GSL I) was used to specifically stain endothelial cells. GSL I was added at dilutions of 1:400 and 1:800 in BSA-PBS and left overnight at 4°C. Negative controls were incubated overnight with no primary antibody, i.e. BSA-PBS alone. Following a PBS wash, the secondary antibody (rabbit, anti-GSL I) diluted 1:150 in BSA-PBS was added at 20°C for 30 min. Following another PBS wash, the tertiary antibody

(goat, anti-rabbit IgG) diluted 1:100 in BSA-PBS was added at 20°C for 30 min. Sections were washed in TBS, placed in levamisole (1:100) for 30 min to remove endogenous phosphatase, followed by another TBS wash. Alkaline phosphatase (Vector Red) were added as per manufacturer's instructions and incubated in the dark for 5-20 minutes until sufficient staining occurred.

Cell nuclei were stained with haematoxylin, washed with acid alcohol (see above), and counterstained with methyl green (1%) for 1 min. Sections were rinsed in distilled water for 30 s, dehydrated and coverslips affixed.

2.11 DRUGS AND REAGENTS

Key

Dilution

k/s: Diluted in Krebs buffer or saline.

DMSO: Diluted in dimethyl sulphoxide.

PBS: Phosphate-buffered saline.

solution: Supplied as a solution.

Storage

day: Stored as solids and dissolved on the day of use/supplied as a solution (stored at 4°C) and diluted on day of use.

week: Diluted and stored as aliquots at 4°C and used within 1 week.

month: Diluted and stored as aliquots at -20°C and used within 1 month.

[1]Powder or aliquot stored under argon

[2]Dissolved immediately before use to prevent oxidation.

[3]Met-haemoglobin was reduced to the ferro-form with sodium dithionite (5-fold excess; 57.4 μM) as described previously (Martin *et al.*, 1985). Dithionite

was then removed by dialysation. Spectrophotometrical analysis at wavelength of 480-620 nm revealed that Hb existed in mostly as oxyhaemoglobin ($HbFe^{2+}O_2$).

Drug	Dilution	Storage	Source
ACh	k/s	month	Sigma
ADP	k/s	month	Sigma
BSA	PBS	day	Sigma
BSO	k/s	day	Sigma
L-Cysteine	k/s	day	Sigma
collagen	k/s	month	Labmedics
DEA/NO	1 M NaOH	month	Alexis
DePeX	solution	day	BDH
DETCA	k/s	month	Sigma
DQ	DMSO	day	Sigma
DTNB	DMSO	day	Sigma
EA	DMSO	day	Sigma
eosin	solution	day	BDH
GSNO	k/s	month	Sigma
GTN (nitrocine)	k/s	month	Schwarz Pharma
GlycoSNAP-2	k/s	month	Alexis
haematoxylin	solution	day	BDH
halothane	solution	day	Genus Express
Hb (from bovine erthrocytes)	H_2O	month[3]	Sigma
heparin (Multiparin)	k/s	day	Genus Express
HQ	k/s	day/month[1,2]	Sigma

Hypnorm	solution	day	Genus Express
$^{111}InCl_3$	k/s	day	NEN™
isoprenaline (isoproterenol)	k/s	day^2	Sigma
Krebs buffer salts	H_2O	day	Fisher
Levamisole	H_2O	day	Sigma
L-NAME	k/s	month	Sigma
MERC	k/s	day	Sigma
ODQ	DMSO	day	Tocris
PE	k/s	week	Sigma
PGI_2	k/s	month	Biomol
RIG200	k/s	month	Synthesised[a]
saline (sterile)	solution	day	Baxter Healthcare
SNP	k/s	month	Sigma
sodium pentobarbitol (Sagatal)	solution	day	Genus Express
SPER/NO	0.01 M NaOH	month	Alexis
SNVP	k/s	month	Synthesised[b]
TESPA	acetone	day	Sigma
Trypsin	H_2O	day	Sigma
Vector Red	solution	day	Vector

All basic laboratory chemicals, e.g. acetone, DMSO, ethanol etc, were supplied by Fisher. All compressed gases were supplied by BOC.

Company locations

Alexis	Nottingham, U.K.

Baxter Healthcare	Glasgow, U.K.
Biomol	Distributed by: Affiniti Research Products, Exeter, U.K.
BDH	Merck Ltd., Lutterworth, U.K.
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Synth: Synthesised in St. Andrews by Dr F.A.Mazzei and Dr A.R. Butler, by a published method [a](Megson *et al.*, 1997), [b](Miller *et al.*, 2000).

2.12 ANALYSIS OF RESULTS

Analysis of the results of individual techniques are given in the method sections of relevant chapters.

P-values quoted in the text were accepted as statistically significant when $P<0.05$. In figures, statistical significance is represented as $*P<0.05$, $**P<0.01$, $***P<0.001$, n.s.=non significant.

Chapter 3

Soluble guanylate cyclase-independent vasodilatation and its relationship to the site of decomposition of NO donors

3. SOLUBLE GUANYLATE CYCLASE-INDEPENDENT VASODILATATION AND ITS RELATIONSHIP TO THE SITE OF DECOMPOSITION OF NO DONORS

3.1 INTRODUCTION

It is generally accepted that the vasodilator effects of NO are mediated via activation of smooth muscle cell sGC to generate cGMP (Waldman & Murad, 1987). However, vasodilatation in response to high concentrations of NO is not exclusively cGMP mediated (Trottier *et al.*, 1998; Weisbrod *et al.*, 1998). Using the selective sGC-inhibitor, ODQ (Garthwaite *et al.*, 1995), an sGC-independent component of vasodilatation has been identified in response to several NO donors (Brunner *et al.*, 1996; Homer *et al.*, 1999). S-nitrosothiols have also been shown to cause vasodilatation in the presence of ODQ, despite complete abolition of cGMP generation (Brunner *et al.*, 1996; Moro *et al.*, 1996).

The identity of the sGC-independent mechanism has not been fully elucidated, although it appears that the mode of NO generation from NO donors influences their actions (Campbell *et al.*, 1996; Homer *et al.*, 1999; Tseng *et al.*, 2000; Wanstall *et al.*, 2001). In the majority of cases, the mechanism of action and site of decomposition of NO donors has yet to be fully determined. The S-nitrosothiols, in particular, can be bioactivated by a number of mechanisms, including decomposition by metal ions (Dicks *et al.*, 1996; Al-Sa'doni *et al.*, 1997; Butler *et al.*, 1998) or cellular enzymes (Kowaluk & Fung, 1990; Freedman *et al.*, 1995; Hou *et al.*, 1996; Trujillo *et al.*,

1998; Jourd'heuil *et al.*, 1999), chemical reduction (Singh *et al.*, 1996; Holmes & Williams, 1998) and transnitrosation of thiols (Park, 1988; Askew *et al.*, 1995). The locations of these factors (e.g. intracellular, extracellular, membrane-bound) and the ability of the S-nitrosothiol to permeate the plasma membrane, will influence the site of release of NO.

The NONOates are a novel class of NO donor drug that decompose by a mechanism that is not catalysed by thiols or biological tissue (Mooradian *et al.*, 1995). Instead, decomposition occurs spontaneously in solution at physiological pH and temperature (Morley & Keefer, 1993), at a rate that is dependent on the nucleophile adduct. (Z)-1-{N-[3-Aminopropyl]-N-[4-(3-aminopropyl-ammonio)butyl]-amino}-diazen-1-ium-1,2-diolate (SPER/NO) contains spermine as the nucleophile and decomposes at a relatively slow rate (half life = 39 min; 37°C, pH 7.4; Maragos *et al.*, 1991). The large size of the spermine adduct suggest that SPER/NO will not gain access to cells. Subsequently, SPER/NO should generate specifically NO, entirely in the extracellular space.

In an effort to clarify the role of sGC-independent vasodilatation and its relationship to the site of NO release, this chapter compares the vasodilator effect of S-nitrosothiols with that of SPER/NO. The use of SPER/NO avoids the difficultly in accurately preparing "authentic" NO solutions that are notoriously difficult to use and tend to generate variable results (Wanstall *et al.*, 2001). In addition, vasodilatation is compared to a number of other NO donor drugs with different release mechanisms in order to test the hypothesis that the sGC-independent activity of NO is exclusive to agents that generate NO outside target smooth muscle cells. Experiments were also designed to test the further hypothesis that sGC-independent vasodilatation is mediated by peroxynitrite.

3.2 METHODS

3.2.1 Preparation

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (250-350 g; n=93) in a perfusion system. Vessels were perfused (0.6 ml min^{-1}) and superfused (1 ml min^{-1}) with fresh oxygenated Krebs buffer solution. Vessel tone was measured by monitoring perfusion pressure with a differential pressure transducer. All experiments were carried out in a darkened laboratory (see Section 2.3.1).

3.2.2 Experimental protocol

Vessels were preconstricted with phenylephrine (PE; 2-14 μM) in the presence of supramaximal concentrations of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (20 μM; See Section 2.3.2). Sequential microinjections of increasing concentrations (10 μl; 10^{-8} - 10^{-3} M) of NO donors were carried out before and after perfusion of modulators. Treatment was restricted to one NO donor and one modulator per vessel. Oxyhaemoglobin (Hb, 10 μM) was used to investigate the role of extracellular NO release (Martin *et al.*, 1985). Hb can be S-nitrosated to form S-nitrosohaemoglobin, a potential NO donor itself (Jia *et al.*, 1996), and is also susceptible to oxidation to *met*Hb by ODQ (Moro *et al.*, 1996). For this reason, it was necessary to use an alternative NO scavenger to facilitate co-perfusion with ODQ. Hydroquinone (HQ; 100 μM) was also used to investigate extracellular NO release, as HQ was confirmed to be a direct NO scavenger in rat vascular tissue (see results). An excess concentration (20 μM) of ODQ was used as a selective inhibitor of sGC (Garthwaite *et al.*, 1995). Endogenous Cu/Zn SOD was inhibited using diethyldithiocarbamic acid (DETCA; 100μM; Misra, 1979; Cocco *et al.*, 1981).

Duroquinone (DQ; 100 μM; Lilley & Gibson, 1995) was perfused, following DETCA pretreatment (Paisley & Martin, 1996), to elevate superoxide levels further, confirmed by experiments using an NO electrode (Sect 3.2.3).

ODQ was perfused for 20 min and then washed out; the irreversible nature (Garthwaite *et al.*, 1995; Hobbs, 1997) of inhibition of sGC with ODQ ensured activity throughout the experiment. DETCA was added to both the perfusate and superfusate for 30 min, and then washed out. Perfusion of Hb, HQ and DQ began 20-30 minutes before administration of NO donors and was continued until the end of the experiment to ensure these drugs were present during application of NO donors. Where Hb, HQ or DQ and ODQ were used together, ODQ was co-perfused only during the first 20 min treatment and Hb, HQ or DQ perfusion was continued alone (see Fig 3.1.).

Following perfusion of modulators, PE concentrations were adjusted to re-establish the baseline pressure of the previous control concentration-response curve, to eliminate pressure change as a possible cause of the subsequent changes in vasodilator amplitude. Following each concentration-response curve, a microinjection (10 μl) of the adenylate cyclase-activator isoprenaline (ISP; 10^{-3} M), was made before and after modulator perfusion, to investigate the effect of the modulator on NO:sGC-independent vasodilation.

3.2.3 NO electrode measurements

An isolated NO electrode was allowed to stabilise (10-30 min) and then was calibrated using DEA/NO (100-800 nM) in phosphate buffer (pH 4; see Section 2.2). Krebs buffer (pH 7.4) was used for subsequent protocols. SPER/NO (10 μM) was introduced into the cuvette and NO generation was allowed to reach plateau, prior to addition of either HQ (100 μM) or DQ (100 μM). Cu/Zn-SOD (250 U/ml) was added to establish the contribution of superoxide in HQ/DQ-induced changes in the NO

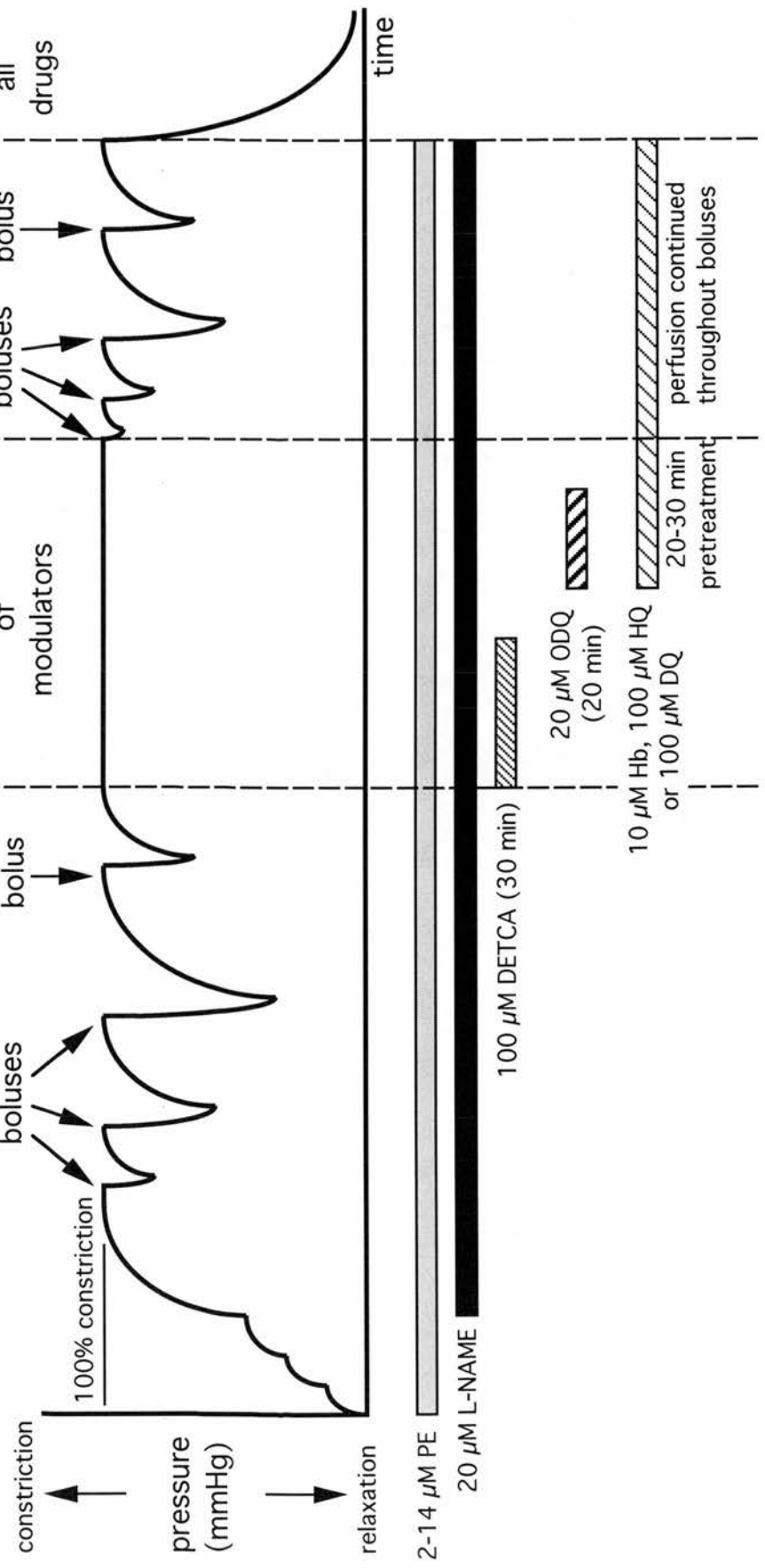


Figure 3.1 Schematic diagram of the experimental protocol used in this study. Note that following a control concentration-response curve, DETCA and ODQ are washed out to prevent cytotoxicity, whereas, Hb, HQ and DQ are perfused continuously till the end of the experiment..

signal. In a second set of experiments, 1 cm segments of rat aorta were homogenised in Krebs buffer (50 µl) using a micropestle. Supernatant (40 µl) was removed and added to cuvettes, before addition of SPER/NO. In several experiments (n=4-8) the aorta was pretreated with DETCA (100 µM) for 30 min and then thoroughly washed in normal Krebs buffer, prior to homogenisation.

3.2.4 Analysis of results

Vasodilator response amplitude was expressed as a % of (PE+L-NAME)-induced pressure existing before drug delivery (% pressure change; positive values represent vasodilatation, where 100% represents complete abolition of agonist-induced tone). Preliminary experiments showed that peak dilatation correlated well with the time course of vasodilatation (area under curve), regardless of the modulator used. Changes in tone induced by perfusion of modulators are expressed as a % of perfusion pressure before drug perfusion.

In NO electrode experiments, maximum response (mV) to known concentrations of DEA/NO, were used to calibrate the electrode. The effect of modulators on NO measurement were then expressed as a % of the SPER/NO response. Mean values are given ± S.E.M..

P-values in the text were obtained by two-factor, repeated measures analysis of variance (ANOVA), unless otherwise stated. Paired and unpaired Student's *t*-tests were all two-tailed. Only key comparisons are indicated in the figures.

3.3 RESULTS

3.3.1 Vessel preconstriction

Vessels were preconstricted with PE (5.0±0.2 μM; n=186) to give pressures of ~50 mmHg (49±2 mmHg; n=186). L-NAME (20 μM) led to a 145±8% increase in pre-existing PE-induced pressure to generate a final pressure of 120±3 mmHg (n=186).

3.3.2 Vasodilator responses to bolus injections of NO donors

Microinjections of NO donors (10 μl; 10^{-8} - 10^{-3} M) produced concentration-dependent, transient vasodilatations in endothelium-intact vessels (Fig 3.2). Following vasodilatation, perfusion pressure recovered fully and was not significantly different from pre-injection pressure ($P>0.05$; paired Student's *t*-test; n=6 for each NO donor). Sequential boluses of NO donors (10 μl; 10^{-8} - 10^{-3} M) had no effect on the magnitude of the response to subsequent microinjections. The maximum response to SNP (10^{-3} M) recovered slowly and, for this reason, a concentration range of 10^{-8} - 10^{-4} M was used. All NO donors exhibited a similar concentration response relationship, with the exception of SPER/NO which was ineffective at concentrations $<10^{-6}$ M. Microinjections of the parent compound, spermine (10 μl; 10^{-6} - 10^{-3} M) had no vasodilator action (n=4).

3.3.3 The effect of modulators on baseline pressure

There was no significant difference in perfusion pressure before and after Hb (10 μM) or ODQ (20 μM) perfusion, separately or when co-perfused ($P>0.05$ for all; n=6, paired Student's *t*-test). Perfusion of DETCA (100 μM) produced a rapid increase in

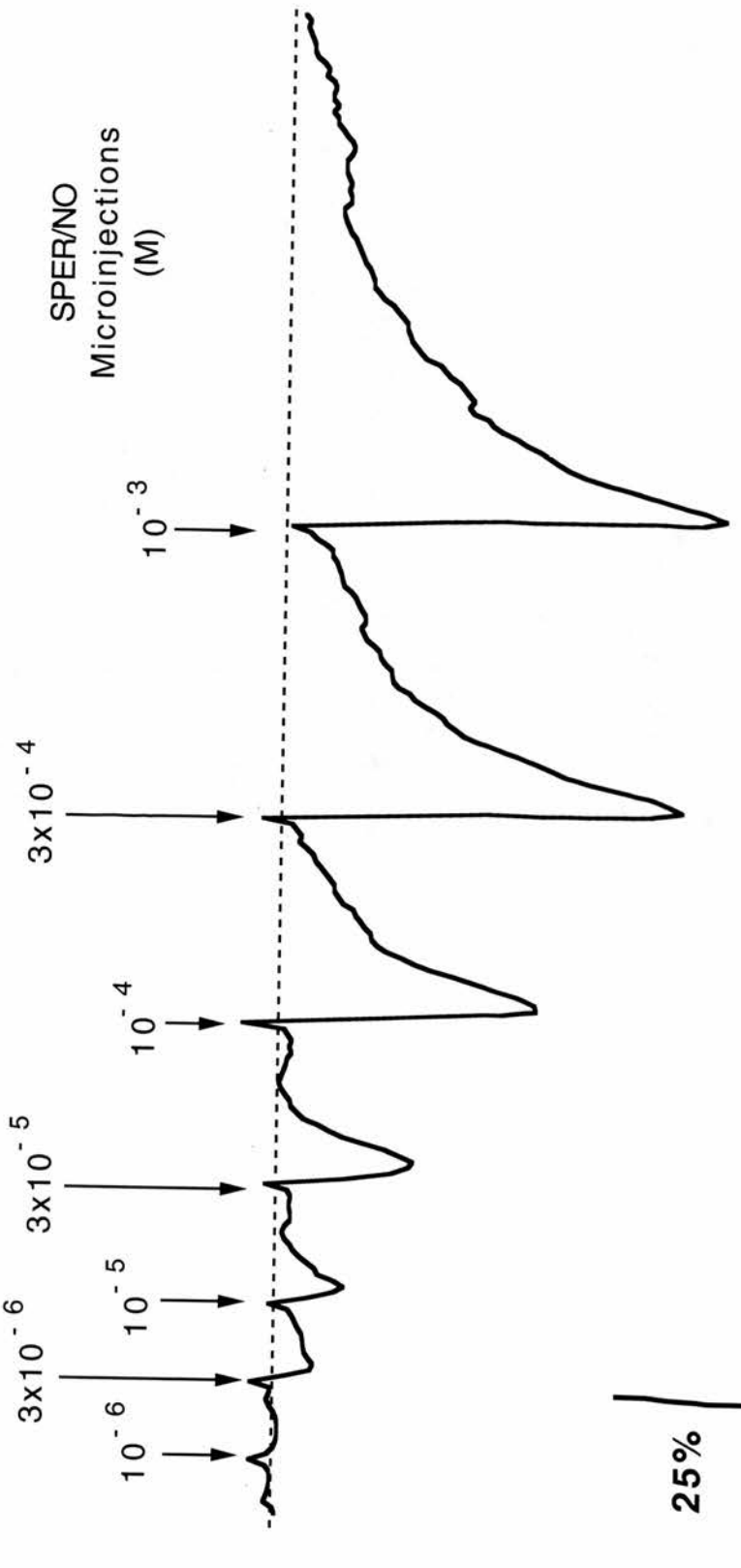


Figure 3.2 Example pressure recordings of vasodilator responses (% preconstriction pressure) to microinjections (10 µl) of SPER/NO (10^{-6}-10^{-3} M). Vessels were preconstricted with PE (2-14 µM) in the presence of L-NAME (20 µM). Preconstriction pressure is represented by top dotted line (lower dotted line represents 100% vasodilatation).

pressure of 26.3±6.5%, which remained significantly greater than baseline after 30 min (P=0.018; n=6; paired Student's t-test). Washout of DETCA resulted in a fall in pressure which often stabilised below baseline levels. HQ (100 μM) produced a significant increase in pressure of 17.5±3.9% (P=0.028; n=6; paired Student's t-test). Preliminary experiments indicated that DQ caused sufficient increase in perfusion pressure to permanently damage the vessel. As a precaution, PE concentration was reduced (x0.5) prior to DQ perfusion, preventing a quantitative measurement of the effect of DQ on vessel tone being made.

3.3.4 NO electrode measurements

SPER/NO (10 μM) generated NO reaching a plateau of ~500 nM in 12-16 min. In the presence of tissue homogenate, the amplitute of the NO signal for SPER/NO was reduced to ~150 nM. HQ (100 μM) all but abolished the NO signal in both the absence and presence of the aortic homogenate (n=6-8; Fig 3.3). This attenuation was not reversed by SOD (250 U/ml). DQ (100 μM) produced a small attenuation (-15.3±3.4%; n=9) of the NO signal from SPER/NO in the absence of tissue homogenate (Fig 3.3). However, in the presence of homogenate, DQ significantly reduced the NO signal to 34.3±5.6% (P<0.001; unpaired t-test; n=10; Fig 3.3). This effect was reversed by SOD (250 U/ml). SOD had no effect on the NO signal in the absence of HQ/DQ, irrespective of the presence or absence of homogenate. Similar results were found using homogenate from DETCA-pretreated aortae (n=8).

3.3.5 The effect of oxyhaemoglobin on responses to vasodilators

Hb (10 μM) produced a significant rightward shift in the concentration response curves for GSNO, D-SNVP and SPER/NO (P=0.021, P=0.001 & P<0.0001,

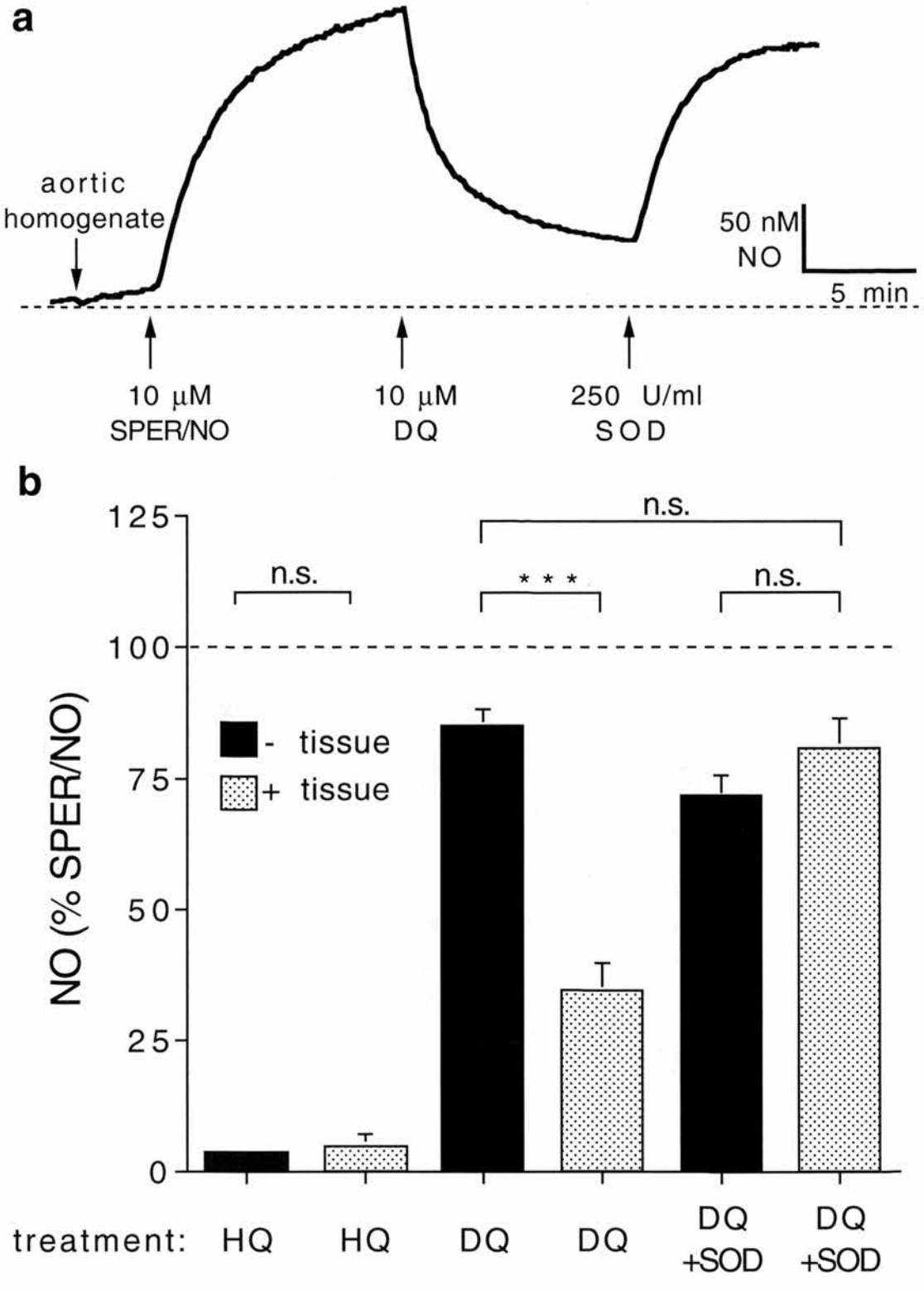


Figure 3.3 Measurement of SPER/NO-derived NO using an NO electrode. a) Example trace of the effect of DQ (100 μM) and SOD (250 U/ml) in the presence of crude aortic homogenate. b) Summary of effect of HQ (100 μM; n=6-8), DQ (100 μM; n=9-10) and SOD (250 U/ml; n=6-10) on NO generation in the presence and absence of tissue. Columns shown are means ± S.E.M. Dotted line represents % maximum NO generated from SPER/NO before drug administration.

respectively; n=6 for all; Fig 3.4a-c). Hb (10 μM) had no significant effect on the response to microinjections of GTN or SNP (P>0.05; n=6; Fig 3.4d,e). Hb also had no significant effect on the response to microinjection (10^{-3} M) of ISP (P=0.52; n=6; one-factor ANOVA; Fig 3.4f).

3.3.6 The effect of HQ on responses to vasodilators

On account of the results from experiments using the NO electrode (Sect 3.3.4), HQ (100 μM) was used as an alternative NO scavenger. HQ (100 μM) had a similar inhibitory effect on SPER/NO-induced vasodilatation in both untreated and DETCA-treated vessels (P=0.20; 2-way factorial ANOVA; n=6-7). Therefore, responses to vasodilators in the presence of HQ in DETCA-pretreated vessels were compared to those in vessels pretreated with DETCA alone (2-way, factorial ANOVA).

Addition of HQ produced a rightward shift in the concentration response curves for GSNO, D-SNVP and SPER/NO (P<0.02; n=6-7; Fig 3.5a-c). In the presence of HQ, responses to high concentrations (>10^{-5} M) of GTN were significantly attenuated (P<0.001; n=6; Fig 3.5d) with a reduction in maximal vasodilatation. DETCA and HQ perfusion had no effect on the response to SNP (P=0.14; n=6; Fig 3.5e), or microinjections (10^{-3} M) of ISP (P=0.48; one-factor ANOVA; n=11; Fig 3.5f).

3.3.7 The effect of DETCA and DQ on responses to vasodilators

DETCA (100 μM) alone had no effect on the response to microinjections of GSNO, SPER/NO, GTN, SNP, or ISP (P>0.05; n=6-7). However, DETCA alone produced a rightwards shift in the dose-response curve to D-SNVP (P=0.005; n=6; Fig 3.6).

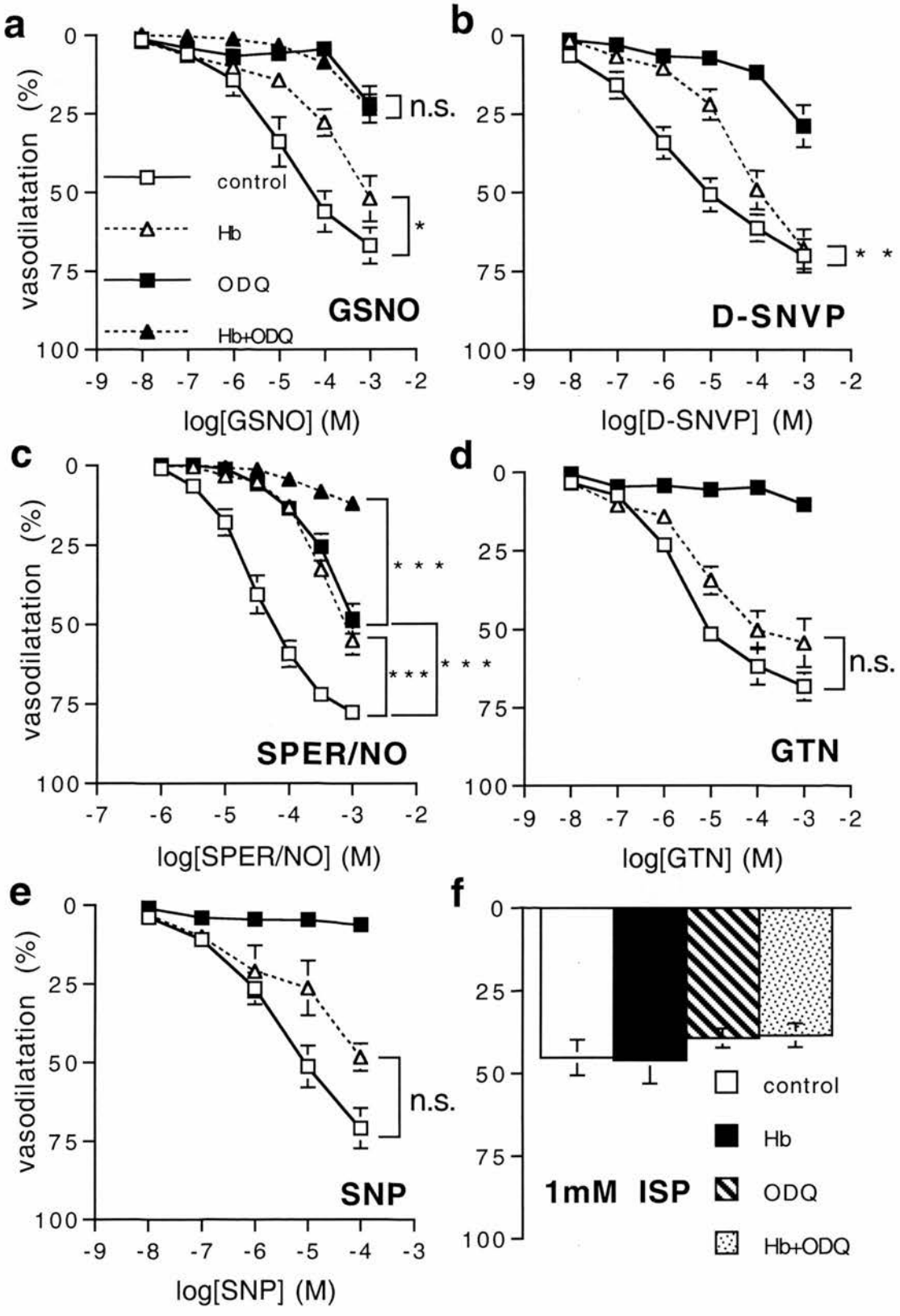


Figure 3.4 Effect of haemoglobin (Hb; 10 μM) and ODQ (20 μM) on vasodilator responses to NO donors (10 μl); (a) GSNO, (b) D-SNVP, (c) SPER/NO, (d) GTN, (e) SNP and (f) ISP. Points shown are means ± S.E.M. (n=6-7).

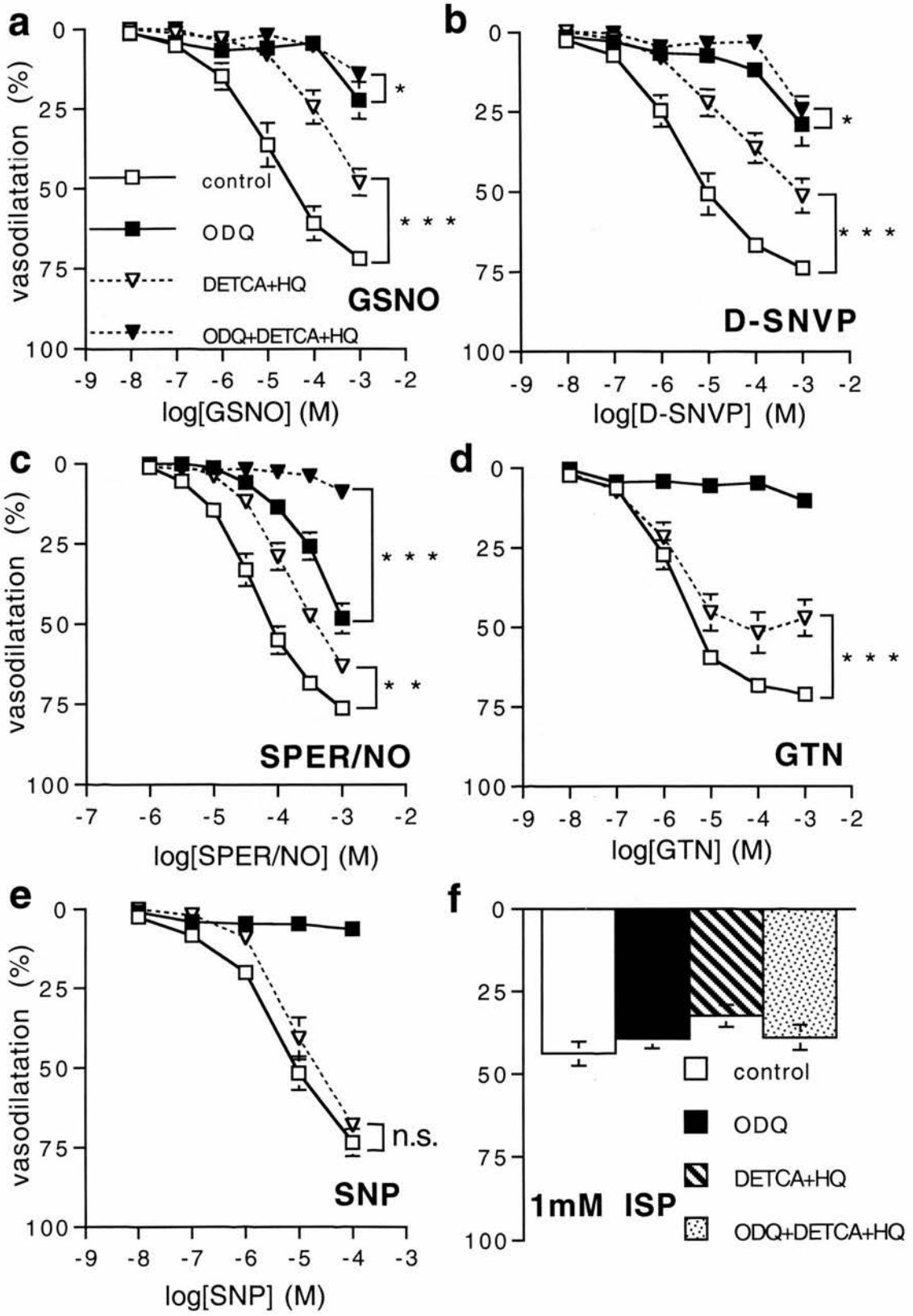


Figure 3.5 Effect of hydroquinone (HQ; 100 μM) and ODQ (20 μM) on vasodilator responses to NO donors (10 μl); (a) GSNO, (b) D-SNVP, (c) SPER/NO, (d) GTN, (e) SNP and (f) ISP. Points shown are means ± S.E.M. (n=6-7).

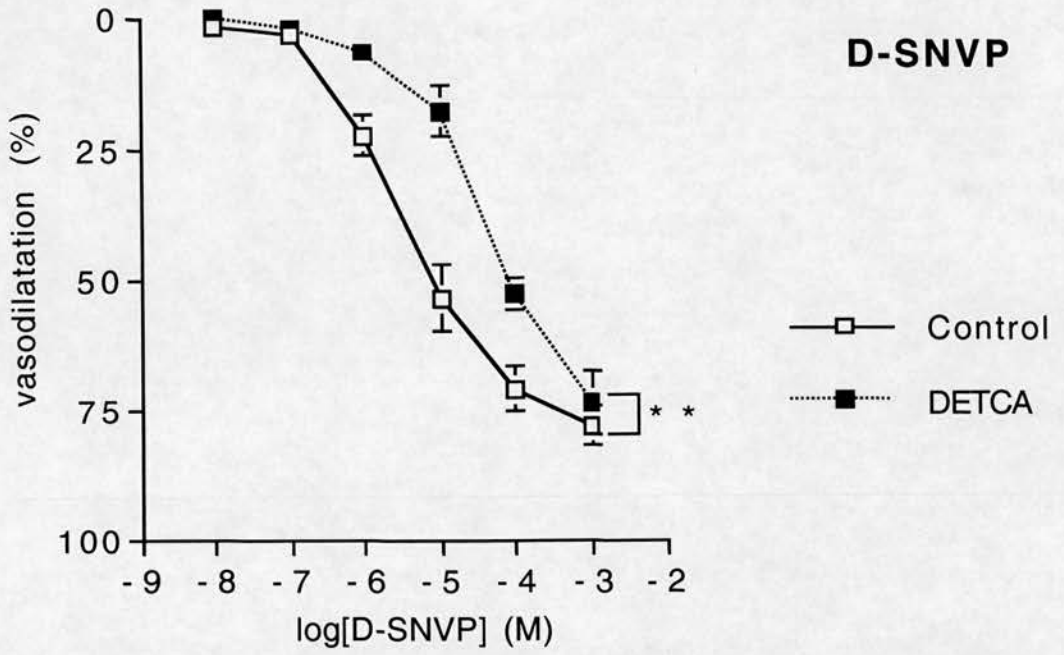


Figure 3.6 Effect of diethyldithiocarbamic acid (DETCA; 100μM) on vasodilator responses to D-SNVP (10 μl). Points shown are means ± S.E.M. (n=6).

DQ (100μM) produced a large attenuation of the responses to all the NO donors tested ($P<0.0001$; n=6-7; Fig 3.7a-e) and greater than the attenuation produced by HQ. DQ also showed a trend to inhibit ISP-mediated relaxation, reducing vasodilatation from 46.8±2.5% to 25.1±3.0%, although this difference was not significant. (10^{-3} M; $P>0.05$; Dunnett's *post-hoc* test, following one-factor ANOVA; n=25: Fig 3.7f). Concentrations of NO donors producing a similar relaxation in control vessels, showed a greater sensitivity to DQ, reducing vasodilatation from ~45% to <15%. In addition, DQ perfusion without DETCA pretreatment still exerted a potent inhibitory effect on vasodilatations to SPER/NO, but had no effect on the vasodilator action of ISP (10^{-3} M; $P>0.05$; Dunnett's *post-hoc* test, following one-factor ANOVA; n=5; Fig 3.7).

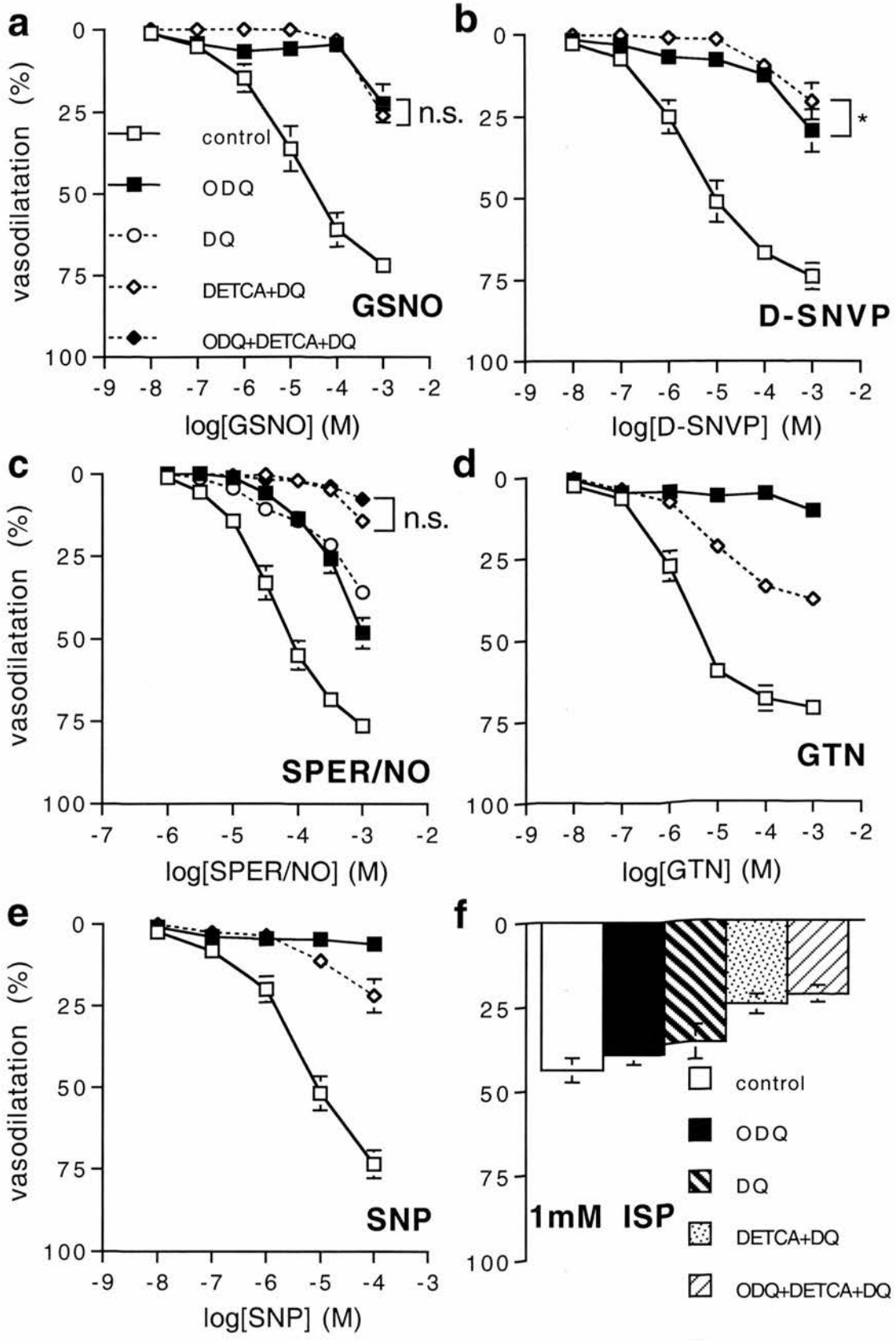


Figure 3.7 Effect of duroquinone (DQ; 100 μM) and ODQ (20 μM) on vasodilator responses to NO donors (10 μl); (a) GSNO, (b) D-SNVP, (c) SPER/NO, (d) GTN, (e) SNP and (f) ISP. Points shown are means ± S.E.M. (n=6-7).

3.3.8 The effect of ODQ on responses to vasodilators

ODQ (20 μM) abolished the response to lower concentrations (<10^{-5} M) of GSNO, D-SNVP and SPER/NO. Higher concentrations of these agents produced vasodilatations that were greatly attenuated compared to control (P<0.004; n=6-7 for all). Responses to the maximum concentration tested (10^{-3} M) were reduced from 68.9±8.1% to 22.4±5.8% for GSNO, 59.0±5.6% to 28.9±6.7% for D-SNVP and 75.0±1.3% to 48.2±4.7% for SPER/NO. ODQ completely abolished vasodilatations to GTN and SNP (P<0.001; n=6-7), but had no effect on the response to microinjection (10^{-3} M) of ISP (P=0.53; paired t-test; n=6).

3.3.9 The effect of NO:sGC modulators and superoxide on ODQ-treated vessels

To investigate the role of extracellular NO in sGC-independent effects, ODQ was co-perfused with Hb (Fig 3.4), HQ (Fig 3.5) or DQ following DETCA pretreatment (Fig 3.7).

Hb, HQ or DQ had little or no effect on ODQ-resistant vasodilatation to GSNO or D-SNVP. HQ and ODQ together produced a small, but statistically significant attenuation of the vasodilatation produced by microinjections of GSNO and D-SNVP, compared to ODQ perfusion alone (P<0.03; 2-way, factorial ANOVA; n=6-7). The maximum response to GSNO (10^{-3} M) was reduced from 22.4±5.8% to 14.2±3.1% and D-SNVP reduced from 28.9±6.7% to 24.2±4.1%.

Co-perfusion of Hb, HQ or DQ in ODQ-treated vessels almost abolished vasodilator responses to SPER/NO, with the maximum response (10^{-3} M) reduced to 12.1±2.1%, 8.8±2.4%, 7.7±2.6% respectively (all P<0.001; n=6). The remaining vasodilatation was not significantly different from responses to SPER/NO in vessels

pretreated with DETCA, ODQ and HQ together (P=0.46; 2-way, factorial ANOVA; n=6).

Microinjections (10^{-3} M) of ISP in the presence of ODQ caused vasodilatations that were not significantly different from those to ISP in the presence of Hb, HQ or DQ alone (10^{-3} M; P>0.05; Dunnett's *post-hoc* test, following one-factor ANOVA; n=11-25).

3.4 DISCUSSION

Here, we show that GTN and SNP release intracellular NO that causes vasodilatation of isolated rat femoral arteries exclusively via activation of sGC. The S-nitrosothiols and SPER/NO are NO donors that can release NO extracellularly and subsequently induce a vasodilatation which is only partially mediated by sGC. The ODQ-resistant vasodilatation of SPER/NO is attenuated by superoxide generation, suggesting that sGC-independent vasodilatation is mediated by NO itself, and is unlikely to require prior generation of peroxynitrite.

3.4.1 Site of NO release from NO donors

These experiments use both Hb and HQ to scavenge extracellular NO. Oxyhaemoglobin is a large protein and will not penetrate cell membranes (Foley *et al.*, 1993). Subsequently, Hb can be considered to be an extracellular scavenger of NO. HQ, a much smaller molecule than Hb, can also be used as an NO scavenger. A number of studies have found that the effects of the HQ are reversed by SOD (Moncada *et al.*, 1986; Kaley *et al.*, 1989; Liu *et al.*, 1994; Paisley & Martin, 1996), indicating that superoxide mediates these actions. Results in other tissues (Hobbs *et al.*, 1991; Lilley & Gibson, 1995; Lefebvre, 1996; La & Rand, 1999), and our NO electrode data (Fig 3.3), show that HQ acts as a direct scavenger of NO, without prior generation of superoxide. In perfused femoral arteries, the effect of HQ was comparable to that of Hb, even following DETCA pretreatment, suggesting that HQ does not generate significant amounts of superoxide. Additionally, the minimal effects of HQ on GTN and SNP (see below), especially in comparison to DQ, suggest HQ acts only as an extracellular scavenger.

A number of mediators are involved in the decomposition of S-nitrosothiols, including metal ions, thiols and enzymes (Megson, 2000). The inhibitory effect of Hb and HQ on the vasodilator actions of both GSNO and D-SNVP suggest that a proportion of NO is released at an extracellular site and that this NO contributes to vasodilatation. D-SNVP is far less susceptible to Cu(I)-mediated decomposition in Krebs buffer than conventional S-nitrosothiols (Megson *et al.*, 1999) and, therefore, it is more likely that tissue components mediate NO release. GSH is a large tripeptide that cannot enter cells intact (Meister, 1984; Li & Trush, 1993). Therefore GSNO is unlikely to gain access to the intracellular environment, but the plasma membrane may mediate its decomposition (Kowaluk & Fung, 1990).

SPER/NO belongs to the diazeniumdiolate (NONOate) class of NO donors (Maragos *et al.*, 1991; Hrabie *et al.*, 1993). At physiological pH, NONOates decompose spontaneously to generate NO without being affected by tissue factors (Morley & Keefer, 1993; Mooradian *et al.*, 1995). SPER/NO is a large molecule and is thought unlikely to gain access into cells, suggesting that the vasodilator action of SPER/NO is primarily through extracellular NO release. Hb and HQ did not abolish the vasodilator action of SPER/NO, suggesting that a proportion of SPER/NO-derived NO enters cells, or reacts with other extracellular factors, before scavenging can occur. It is also possible that a proportion of SPER/NO-derived NO is generated intracellularly and is therefore inaccessible to Hb and HQ. However, a mechanism by which SPER/NO can cross plasma membranes has yet to be described.

Hb did not significantly attenuate the vasodilator actions of GTN, supporting intracellular breakdown of this drug in target smooth muscle cells (Bennett *et al.*, 1989; Chung & Fung, 1990; Schror *et al.*, 1991). Release of NO from SNP is often considered to be spontaneous but SNP does not decompose at physiological pH unless thiols, other reducing agents and mammalian tissue are present (Butler & Glidewell, 1987; Bates *et al.*, 1991; Kowaluk *et al.*, 1992; Butler & Megson, 2002). The lack of effect of Hb and HQ on the vasodilatation of SNP also implies intracellular

decomposition. Nitroprusside is a divalent ion and, although a mechanism by which SNP can cross the plasma membrane has not been speculated on, SNP has been shown to cross the plasma membrane, albeit slowly (Rodkey & Collison, 1977; Butler *et al.*, 1988).

3.4.2 sGC-independent vasodilatation and site of NO generation

Experiments using the selective sGC inhibitor, ODQ, show that several NO donors, SPER/NO in particular, have sGC-independent actions. Previous studies have shown that NO donors are still able to cause vasodilatation (Brunner *et al.*, 1996; Plane *et al.*, 1998; Homer & Wanstall, 2000) or inhibit platelet aggregation (Gordge *et al.*, 1998) even when increases in cGMP levels are abolished. In all of these studies, the concentration of ODQ was lower (1-10 μM) than the concentration used here (20 μM). The lack of effect of ODQ, Hb and HQ on the AC-mediated vasodilator action of ISP, demostrates the specificity of these drugs to the NO:sGC pathway and lack of toxicity at the concentrations used.

The different mechanisms of action of endogenous NO and different NO donors in blood vessels has been addressed recently (Tseng *et al.*, 2000; Wanstall *et al.*, 2001) in studies that focus on the role of other redox forms of NO (NO^-, NO^+) in vasodilatation. The study concluded that redox status was not the main determinant of sGC involvement. Homer *et al* (Homer *et al.*, 1999) made the observation that NO donors that require intracellular metabolism to generate NO (GTN, isosorbide dinitrate and SNP), induce vasodilatation that is abolished by ODQ, whereas NO donors that do not require tissue-activation to generate NO (SIN-1, NONOates) exhibit a sGC-independent component of vasodilatation. The authors also demonstrate that the rate of NO generation does not influence sGC-independent vasodilatation. The present findings agree with these authors, although the results presented here suggest that the

site of NO generation is more likely to influence sGC-independent mechanisms than requirement for metabolism.

ODQ abolished the vasodilator actions of both S-nitrosothiols at low concentrations, although a small vasodilatation was produced at high concentrations. However, S-nitrosothiols are capable of generating both NO in solution as well as tissue derived NO (Megson, 2000). To clarify the role of extracellular NO, we investigated the vasodilator effect of SPER/NO. To the best of our knowledge, this compound should generate NO only in the extracellular space (see above). Of all the NO donors, SPER/NO was able to produce the greatest vasodilatation in ODQ-treated tissues. Spermine itself has been shown to be hypotensive, although in general, spermine does not cause vasodilatation (Maragos *et al.*, 1991). In the present study, spermine was found to be inactive and was therefore unlikely to account for sGC-independent vasodilatation in response to SPER/NO.

Vasodilator responses to both GTN and SNP were abolished by the selective sGC-inhibitor, ODQ, suggesting that NO released intracellularly by these agents induces vasodilatation exclusively via a sGC-mediated pathway. It is likely that the close proximity of the sGC to the site of NO release from GTN/SNP, and rapid binding to the haem group, accounts for all the NO generated, although it is also possible that the intracellular milieu lacks critical substituents necessary to mediate sGC-independent actions.

3.4.3 Reactions of NO in the extracellular space

There are two possible reactions of NO in the extracellular space. Firstly, it can react with superoxide to form peroxynitrite (Mayer *et al.*, 1998; Vinten-Johansen, 2000; Eqn 1).

$$NO + O_2^- \rightarrow ONOO^- \quad (1)$$

Concentrations of superoxide are negligible in healthy vascular tissue due to the activity of antioxidant systems (Freedman & Crapo, 1982). However, Krebs buffer contains glucose and trace metal ions, and subsequently, superoxide may be generated due to the continuous bubbling of high concentrations of oxygen (Gillespie & Sheng, 1990; Beckman & Koppenol, 1996). Secondly, NO reacts with molecular oxygen to form higher nitrogen oxides (Eqn 2,3).

$$2NO + O_2 \rightarrow NO_2 \quad (2)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (3)$$

The reaction of NO with molecular oxygen is slower and second order with respect to NO (Ford *et al.*, 1993; Kharitonov *et al.*, 1995; Keshive *et al.*, 1996). However, the reaction could be significant in oxygen saturated Krebs buffer, when concentrations of NO reach micromolar levels (Beckman & Koppenol, 1996). Therefore, both reactions are plausible, especially at the high concentrations of NO coinciding with the concentrations of NO donors that produce sGC-independent vasodilatation.

3.4.4 Role of peroxynitrite in the vasodilatation to NO donors

To investigate the involvement of peroxynitrite in the vasodilator activity of NO donors, superoxide levels were enhanced using DQ. High levels of superoxide might affect the activity of NO-releasing compounds in several ways. Firstly, superoxide will scavenge free NO released from donors and prevent it from reacting with sGC. Secondly, the reaction of NO with superoxide will generate significant amounts of peroxynitrite, which in turn is believed to regulate cellular function through the oxidation of biological molecules or through the nitration of tyrosine-containing proteins (White *et al.*, 1994; Beckman & Koppenol, 1996; Vinten-Johansen, 2000). Nitration of tyrosine residues in proteins may affect phosphorylation processes and

modify cellular transduction pathways (Naseem *et al.*, 1997; Squadrito & Pryor, 1998). Thirdly, the rapid reaction rate of NO with superoxide will compete with the reaction of NO with molecular oxygen, limiting the production of higher oxides of nitrogen.

Inhibition of Cu/Zn SOD was combined with a superoxide generating system to enhance superoxide. Firstly, DETCA was used to inactivate the Cu/Zn-SOD that dismutates superoxide in the cytoplasm. It should be noted that DETCA is a non-selective inhibitor of copper-containing compounds and will indirectly affect a number of cellular enzymes (Cocco *et al.*, 1981; Kelner *et al.*, 1989; De Man *et al.*, 1996). The concentration and period of DETCA perfusion were minimised to limit indirect actions (Lilley & Gibson, 1995; Lefebvre, 1996). DETCA had no effect on the vasodilator response to the adenylate cyclase-activator ISP, suggesting that any non-specific effects of DETCA are limited to the NO:sGC pathway. Interestingly, DETCA pretreatment inhibited the vasodilator action of D-SNVP (Fig 3.6). Because DETCA is not present in the perfusate during administration of D-SNVP, it must permanently inhibit part of the pathway needed for D-SNVP to induce vasodilatation. Unlike GSNO, which is not inhibited be DETCA pretreatment, D-SNVP is lipophilic and is more likely to cross the plasma membrane. In the intracellular environment, D-SNVP will undergo transnitrosation reactions, passing its nitrosonium group onto other thiols, such as GSH or cysteine. Both the resulting nitrosated thiols, GSNO and SNOC, are more susceptible to copper-mediated breakdown than D-SNVP. Therefore, DETCA could inhibit the vasodilator activity of D-SNVP by preventing the Cu-mediated release of NO from intracellular S-nitrosothiols, formed by transnitrosation reactions with D-SNVP.

Superoxide levels were further enhanced, using DQ as an exogenous superoxide generator. DQ auto-oxidises very slowly in solution, but in the presence of tissue factors, superoxide is formed (Rossi *et al.*, 1986; Boersma *et al.*, 1994). Therefore, DQ was expected to generate superoxide in the close vicinity of tissue.

Indeed, the electrode experiments showed that DQ could only reduce the amount of detectable SPER/NO-derived NO in the presence of tissue; an effect that was reversed by SOD (Fig 3.3). In femoral arteries, the vasodilator response to SPER/NO was attenuated by DQ perfusion, especially in DETCA-pretreated arteries. This suggests that DQ generates superoxide in femoral arteries that has access to the intracellular environment (Lynch & Fridovich, 1978; Mao & Poznansky, 1992).

Similar to the findings of others, (Ignarro *et al.*, 1988; Hobbs *et al.*, 1991; Alsip & Harris, 1992; Hussain *et al.*, 1996), GTN was more resistant to superoxide generation than other NO donors. In particular, superoxide generation produced a reduction in the maximum response to GTN. This type of inhibition would not arise from the scavenging of NO by superoxide, but more likely reflects an inhibition of the bioconversion of GTN to NO (Servent *et al.*, 1989; Laight *et al.*, 1997; Hanspal *et al.*, 2002). Bioconversion of GTN and subsequent release of NO both occur intracellularly, providing additional evidence that a significant proportion of the action of DQ occurs at an intracellular site. The DQ-induced increase in perfusion pressure and the small inhibitory effect on ISP, suggest that DQ has some actions that are not related to NO scavenging. However, increasing superoxide levels in the absence of SOD will unavoidably affect other antioxidant systems, including those related to glutathione. Thiols like glutathione have been shown to be involved in ISP-induced vasodilatation (Needleman *et al.*, 1973) and GTN-metabolism (Ignarro *et al.*, 1981; Feelisch & Noack, 1987; see Chapter 4).

DQ further attenuated the sGC-independent vasodilatation of GSNO, D-SNVP and SPER/NO in ODQ-treated vessels. Under these conditions, superoxide will rapidly react with NO to form peroxynitrite (Beckman & Koppenol, 1996; Squadrito & Pryor, 1998). Because superoxide generation did not potentiate the responses to NO donors in the presence of ODQ, it is unlikely that peroxynitrite mediates sGC-independent vasodilatation.

3.4.5 Potential NO-mediated sGC-independent mechanisms

The sGC-independent actions of SPER/NO were almost abolished when vessels were perfused with an extracellular NO scavenger (Hb, HQ, DQ-derived superoxide). We hypothesise that at high concentrations of SPER/NO, sufficient NO is released extracellularly to react with molecular oxygen. This reaction can form nitrosating species that interact with sulphydryl-containing molecules (Kharitonov *et al.*, 1995; Keshive *et al.*, 1996). The sGC-independent actions of S-nitrosothiols, although small in comparison to those of SPER/NO, were less susceptible to inhibition by extracellular NO scavengers. However, these compounds can nitrosate sulphydryl groups directly through transnitrosation reactions without the release of free NO (Park, 1988; Askew *et al.*, 1995). Nitrosation of thiol-containing residues in enzymes has been shown to regulate their function (Stamler *et al.*, 1992; Lipton *et al.*, 1993; Bolotina *et al.*, 1994; Clementi *et al.*, 1998; Bauer *et al.*, 1999; Xie *et al.*, 1999). In addition, modifiers of protein sulphydryl groups inhibit NO-mediated changes in the activity channels (Bolotina *et al.*, 1994; Campbell *et al.*, 1996). Therefore, S-nitrosation of thiol-containing proteins on the extracellular surface of the plasma membrane, either by the reaction of extracellular NO with oxygen or direct transnitrosation reactions, represent a plausible sGC-independent mechanism. Plasma membrane ion channels are the most likely candidates, specifically voltage-sensitive calcium channels (Travis *et al.*, 2000) and calcium-dependent potassium channels (Mistry & Garland, 1998; Plane *et al.*, 1998; Homer & Wanstall, 2000).

3.4.6 Physiological relevance of data

Further work is needed to ascertain the relative importance of these mechanisms *in vivo*. One consideration is whether oxygen concentrations *in vivo* are sufficient to interact with physiological concentrations of NO. This could be investigated in the same experimental set-up by reducing oxygenation of the perfusate by bubbling with argon. However, because the reaction of NO with O_2 is second order with respect to NO, oxygen is unlikely to be the rate limiting factor (Beckman & Koppenol, 1996). Indeed, superoxide generation was used as a competitive inhibitor of this reaction, as it is unlikely that the level of oxygen in the Kreb's solution could be lowered to such an extent that the NO/O_2 reaction could be significantly reduced without producing tissue hypoxia.

Another consideration is whether physiological concentrations of NO are high enough to produce sGC-independent effects. Levels of circulating S-nitrosoalbumin have been estimated to be in the micromolar range (Stamler *et al.*, 1992) (although, this may be an overestimation; Marley *et al.*, 2001). However, S-nitrosothiol-induced the sGC-independent vasodilatation is small even at high concentrations and, therefore, are unlikely to significantly influence vessel tone. A far more dramatic sGC-independent component of S-nitrosothiol-mediated inhibition of platelet aggregation has been linked to the proportion of NO released extracellularly by these agents (Sogo *et al.*, 2000). It is possible that the sizeable sGC-independent actions of S-nitrosothiols account for the platelet selectivity of these compounds (De Belder *et al.*, 1994).

The extracellular NO generator SPER/NO produced a sizeable sGC-independent vasodilatation. This sGC-independent vasodilatation may be mirrored by endothelial derived NO as it passes across the extracellular space to VSMCs or by application of pharmacological doses of NONOates. Finally, it should be noted that the contribution of NO-related sGC-independent pathways may be upregulated in cardiovascular conditions, such as hypercholesterolaemia (Najibi *et al.*, 1994) or

inflammatory conditions where iNOS is expressed (Davies *et al.*, 1996; Hobbs *et al.*, 1999; see Sect 1.7).

3.4.7 Summary

SPER/NO releases NO extracellularly and induces a vasodilatation that is entirely dependent on NO, but not exclusively mediated by sGC. S-nitrosothiols are also capable of releasing extracellular NO and produce a small sGC-independent vasodilatation, although only at high concentrations. The sGC-independent vasodilatation of these compounds are not augmented, indeed they are attenuated, by superoxide generation, suggesting that these effects are not mediated by peroxynitrite. We suggest that sGC-independent vasodilatation may be mediated through the reaction of NO with molecular oxygen, forming higher nitrogen oxides which can regulate the function of thiol-containing proteins (e.g. ion channels) through S-nitrosation (Fig 3.8). Alternatively, the S-nitrosothiols may be able to regulate protein function, by transnitrosation reactions, without the release of free NO.

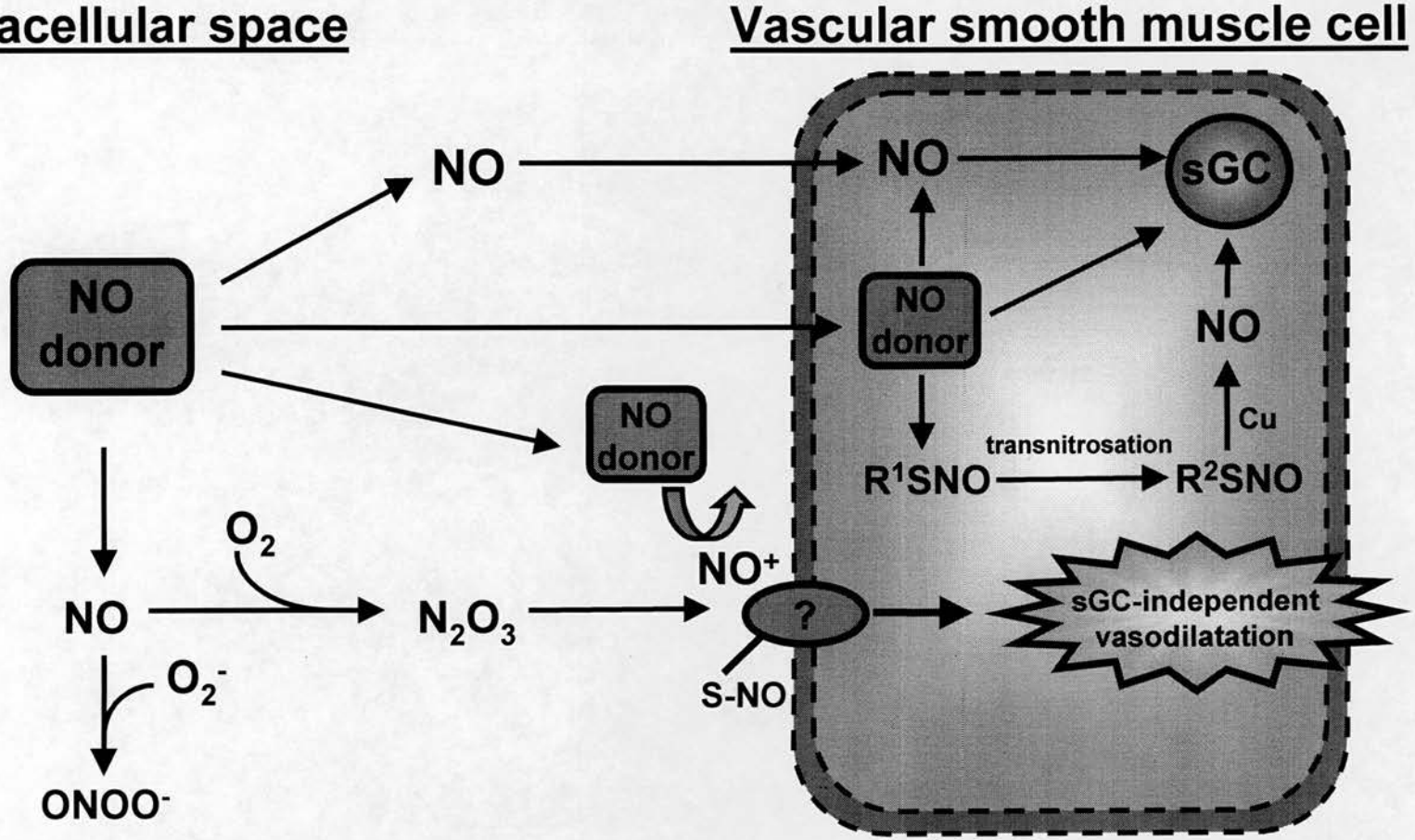


Figure 3.8 Schematic diagram of the possible mechanism of action of the NO donors used in this study. Some NO donors enter VSMCs, decompose to NO and stimulate sGC (i.e. GTN and SNP). D-SNVP may also pass into cells and undergo transnitrosation reactions with intracellular thiols, that generate NO via Cu-mediated catalysis. NO may be generated in the extracellular space (i.e. SPER/NO, GSNO & D-SNVP), a portion of which will pass directly into VSMCs. Instead, extracellular NO may react with superoxide to form peroxynitrite, which does not possess vasodilator activity in this tissue. NO may also react with molecular oxygen to form higher oxides of nitrogen which are capable of S-nitrosating thiol-containing proteins. This process may be linked to sGC-independent vasodilatation. S-nitrosothiols (GSNO and D-SNVP) can also directly nitrosate thiols without the release of free NO.

Chapter 4

The role of thiols in the vasodilator action of NO donor drugs

4. THE ROLE OF THIOLS IN THE VASODILATOR ACTION OF NO DONOR DRUGS

4.1 INTRODUCTION

The cells of the cardiovascular system, like other organs, contain high concentrations of thiols. In VSMCs, endothelial cells, platelets and red blood cells, the low molecular weight thiol glutathione (GSH) is present in almost millimolar concentrations (0.5-10 mM). Intracellular levels of another important low molecular weight thiol, cyst(e)ine (Cys), are lower, but still significant (5-130 μM) (Griffith, 1981; Gruetter & Lemke, 1985; Thomas *et al.*, 1985; Boesgaard *et al.*, 1993; Mayer *et al.*, 1995; Haj-yehia & Benet, 1996; Mills & Lang, 1996; Gladwin *et al.*, 2000). Additionally, many proteins contain cysteine residues with reduced SH groups, including ion channels and enzymes (Upchurch *et al.*, 1995; Gaston, 1999). Thiols are also found in extracellular fluids; blood plasma contains high concentrations of both low molecular weight thiols and larger protein thiols such as albumin (2-20 μM & 300-750 μM, respectively) (Peters, 1985; Mansoor *et al.*, 1992; Boesgaard *et al.*, 1993; Haj-yehia & Benet, 1996; Jones *et al.*, 2000; Jourd'heuil *et al.*, 2000; Marley *et al.*, 2001; Tsikas *et al.*, 2001). In all cells, thiols and particularly GSH, form a major part of the anti-oxidant system, regulating the redox state of intracellular molecules and protecting against physiological and pathophysiological formation of oxygen free radicals (Meister, 1994; Griffith, 1999). Thiols, therefore, influence NO bioactivity by protecting NO from scavenging by reactive oxygen species. However, they have also been shown to have a number of other functions that impact on NO bioactivity, including the sequestration of NO in the

form of S-nitrosothiols and the regulation of enzymes like guanylate cyclase (Upchurch *et al.*, 1995; Gaston, 1999; Moore & Mani, 2002).

The large size and polarity of many NO donors will restrict their passage across the plasma membrane, preventing them from reaching their intracellular targets. In general, it is assumed that these drugs decompose to generate NO, which can freely diffuse into cells. Interestingly, the vasodilator potency of S-nitrosothiols varies little despite considerable differences in size, stability and lipophilicity (Kowaluk & Fung, 1990; Mathews & Kerr, 1993). Therefore, it is unlikely that decomposition in extracellular solution is entirely responsible for their vasodilator action. S-Nitrosothiols can pass their nitrosonium group (NO^+) on to endogenous thiol groups without the release of free NO (Park, 1988; Askew *et al.*, 1995). Subsequently, it has been suggested that S-nitrosothiols use these transnitrosation reactions to pass NO^+ to cell surface thiols and that this is the initial step in the passage of S-nitrosothiol-derived NO across the plasma membrane (Scharfstein *et al.*, 1994; Zai *et al.*, 1999).

Thiols have also been implicated in the metabolism of most NO donors (see Section 1.8), with the only likely exceptions being SIN-1 (Feelisch *et al.*, 1989) and the NONOates (Morley *et al.*, 1993). In particular, intracellular thiols have been proposed as a strict requirement for the metabolism of organic nitrates (Ignarro *et al.*, 1981). Often this proposal is based on the findings of studies that pharmacologically supplement intracellular thiol levels or deplete them using alkylators or oxidising agents (Fung *et al.*, 1989; Lau & Benet, 1992; Boesgaard *et al.*, 1993; De Man *et al.*, 1996; Haj-yehia & Benet, 1996). This was first demonstrated in 1973 by Needleman *et al*, using ethacrynic acid (EA) as a thiol alkylator (Needleman *et al.*, 1973). EA inhibited the vasodilator effects of GTN, however, it is often overlooked that a non-specific inhibition of a wide range of other vasodilators was also observed (Needleman *et al.*, 1973). In almost 30 years there have been numerous pharmacological studies that manipulate thiol levels, and yet it is still not clear whether thiols are specifically

required to biotransform NO donors or whether their main role is downstream of NO release, through the protection of NO and target enzymes from oxidative stress.

This chapter aims to clarify the contradictory literature, by testing the effect of different thiol inhibitors on the vasodilatation of a range of NO donors with different NO release mechanisms. Responses to the adenylate cyclase (AC) activator, isoprenaline (ISP) are also tested, to investigate the effect of thiol inhibition on NO:sGC-independent vasodilatation. It is hypothesised that oxidation of cell surface thiols will have an inhibitory effect on vasodilatation in response to S-nitrosothiols, regardless of their ability to penetrate cell membranes. However, GTN and SNP, which undergo intracellular metabolism (see Chapter 3), will not be affected by the availability of cell surface thiols. Additionally, it is hypothesised that depletion of intracellular thiols, will inhibit the vasodilatation of all NO donors, not just GTN, through loss of antioxidant protection.

4.2 METHODS

4.2.1 Preparation

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (250-350 g; n=73) in a perfusion system. Vessels were perfused (0.6 ml min^{-1}) and superfused (1 ml min^{-1}) with fresh oxygenated Krebs buffer solution. Vessel tone was measured by monitoring perfusion pressure with a differential pressure transducer. All experiments were carried out in a darkened laboratory (see Section 2.3.1).

4.2.2 Experimental protocol

Vessels were preconstricted with phenylephrine (PE; 2-14 μM) in the presence of supramaximal concentrations of the NO synthase inhibitor L-NAME (20 μM; see Section 2.3.2). Sequential microinjections of increasing concentrations (10 μl; 10^{-8} - 10^{-3} M) of NO donors were carried out before and after pretreatment of modulators of endogenous thiols. Treatment was restricted to one NO donor and one modulator per vessel. 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; 100 μM) was used to block extracellular SH groups (Ellman, 1959; Tang & Aizenman, 1993). Buthionine sulfoximine (BSO; 100 μM; Griffith & Meister, 1979), a cell permeable inhibitor of the rate-limiting enzyme which mediates the *de novo* synthesis of GSH, γ-glutamylcysteine synthase, was used to deplete tissue of the free GSH. The availability of both free thiols and the thiol groups of proteins was inhibited by perfusion with the cell permeable thiol alkylator, ethacrynic acid (EA; 100 μM; Daniel *et al.*, 1971).

Due to the long-lasting inhibitory effects of DTNB and EA, these compounds were perfused for 20 min and then washed out to prevent any direct chemical reactions with NO donors (Fig 4.1a). BSO was perfused for 20 hours to deplete GSH as it is

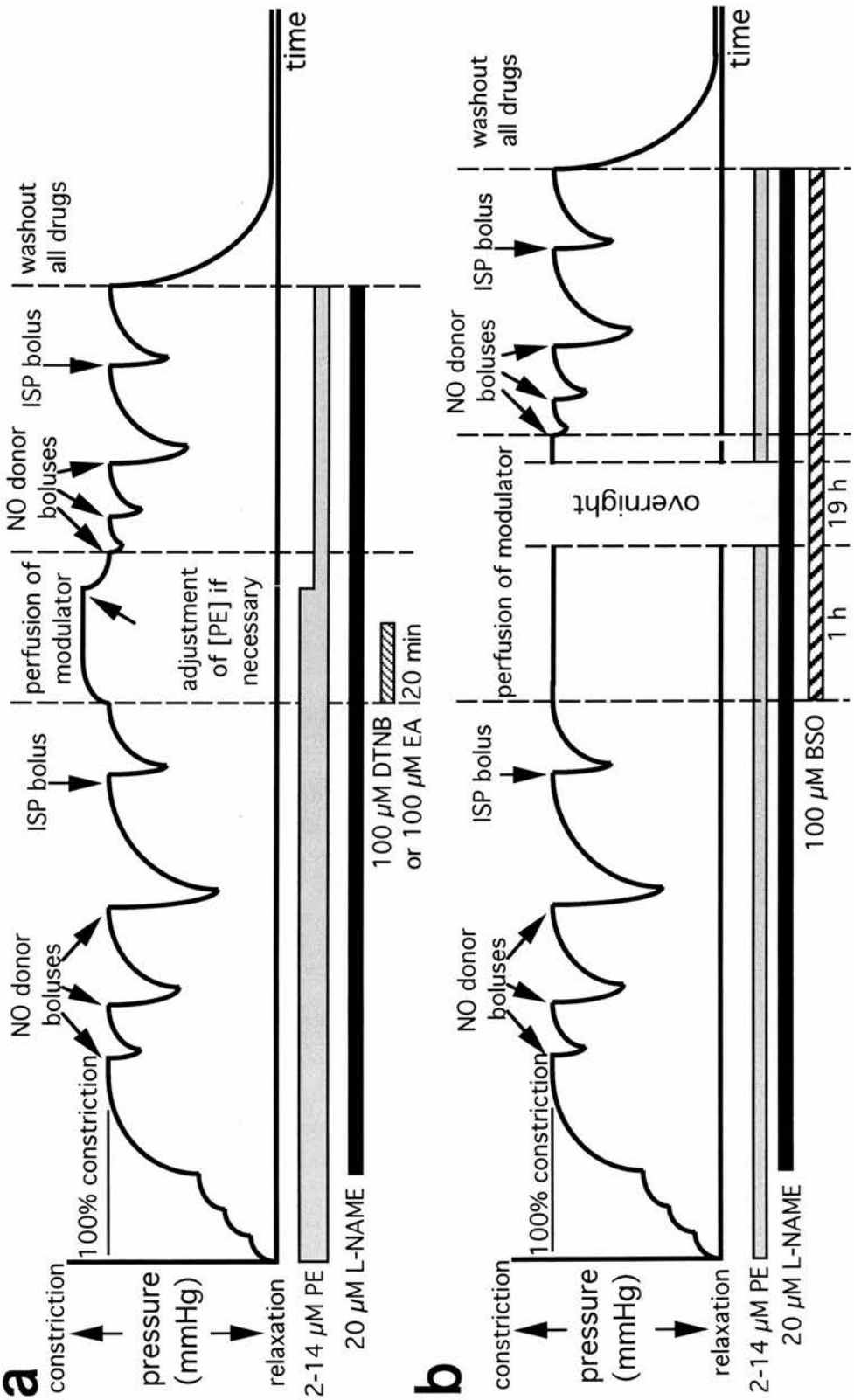


Figure 4.1 Schematic diagram of the experimental protocols used in this study. Note that following a control concentration-response curve, DTNB and EA are washed out to prevent cytotoxicity and direct reaction with drug boluses (a). BSO is perfused for 1 h in the presence of PE and then 19 h the absence of PE to prevent desensitisation. At t=20 h the vessels are perfused with the original solution containing PE and BSO which are present throughout the rest of the experiment (b).

used in cellular functions and exported from cells (see discussion). Vessels were perfused with BSO and perfusion pressure was monitored for 1 h. PE was then removed from the internal perfusate and the perfusion rate lowered to 0.1 ml min^{-1} overnight, at 25°C, to optimise vessel survival. At t=20 h, the original PE-containing solution was re-perfused at the original flow rate (0.6 ml min^{-1}), at 37°C. Unlike, DTNB and EA, BSO was retained in the perfusate during administration of boluses to ensure continued inhibition of GSH synthesis (Fig 4.1b; Griffith, 1999).

Following perfusion of modulators, PE concentrations were adjusted to re-establish the baseline pressure of the previous control concentration-response curve, to prevent pressure change from influencing the amplitude of responses to bolus injections. Following each concentration-response curve, a microinjection (10 µl) of the adenylate cyclase-activator isoprenaline (ISP; 10^{-3} M; ~EC_{50}), was made before and after modulator perfusion, in order to investigate the effect of the modulator on NO:sGC-independent vasodilatation.

4.2.3 Analysis of results

Vasodilator response amplitude was expressed as a % of (PE+L-NAME)-induced pressure existing before drug delivery (% pressure change; positive values represent vasodilatation, where 100% represents complete abolition of agonist-induced tone). Changes in tone induced by perfusion of modulators are expressed as a % of perfusion pressure before drug perfusion. Mean values are given ± S.E.M..

P-values in the text were obtained by two-factor, repeated measures analysis of variance (ANOVA), unless otherwise stated. Paired and unpaired Student's *t*-tests were all two-tailed.

4.3 RESULTS

4.3.1 Vessel preconstriction

Vessels were preconstricted with PE (5.4±1.9 μM) to give pressures of ~50 mmHg (51±2 mmHg; n=142). L-NAME (20 μM) led to a 135±7% increase of pre-existing PE-induced pressure to generate a final pressure of 120±3 mmHg (n=142).

4.3.2 Vasodilator responses to bolus injections of NO donors

Microinjections of NO donors (10 μl; 10^{-8} - 10^{-3} M) produced concentration-dependent, transient vasodilatations in endothelium-intact vessels. All the NO donors used produced vasodilatation over a similar concentration range (Fig 4.2). There was a small, but significant difference between the vasodilatations to D-SNVP and L-SNVP (P<0.001; two-way, unrelated ANOVA; n=20).

4.3.3 The effect of modulators on baseline pressure

DTNB (100 μM) and BSO (100 μM) did not cause any significant change in baseline pressure over their perfusion period (P=0.14 and P=0.80, respectively; paired t-test; n=6), whereas EA (100μM) produced an increase in pressure of 49.9±9.2% (P=0.003; n=6; paired t-test).

4.3.4 The effect of DTNB on responses to vasodilators

DTNB (100 μM) had no significant effect on the response to microinjections of any of the vasodilators tested (P>0.27 for all; n=6-11; Fig 4.3).

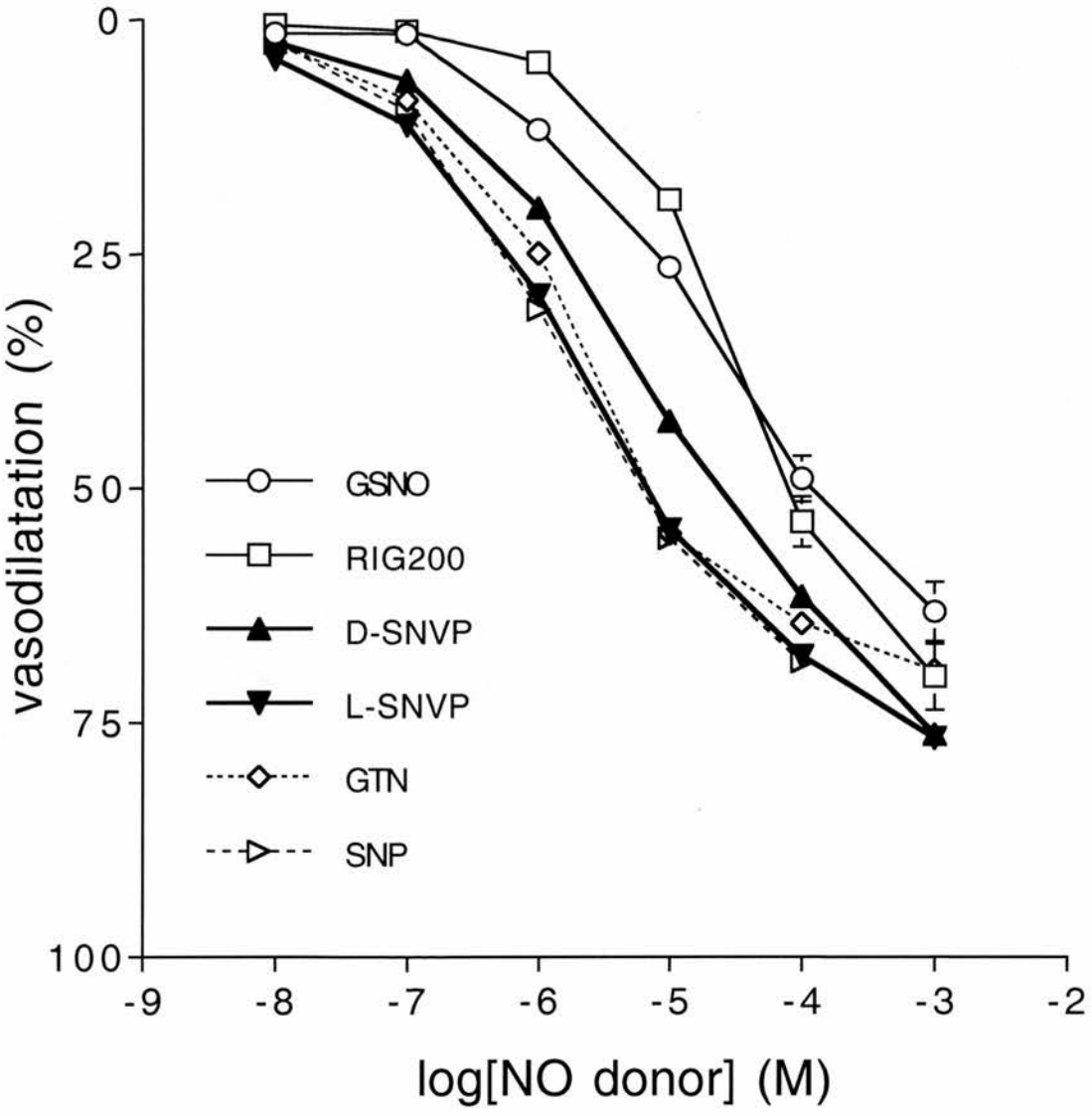


Figure 4.2 Log concentrations response curves to vasodilators (10 µl). GSNO (circles; n=23), RIG200 (squares; n=18), D-SNVP (filled triangles pointing up; n=20), L-SNVP (filled triangles pointing down; n=20), GTN (diamonds; n=21) and SNP (open triangles; n=24). Points shown are means ± S.E.M..

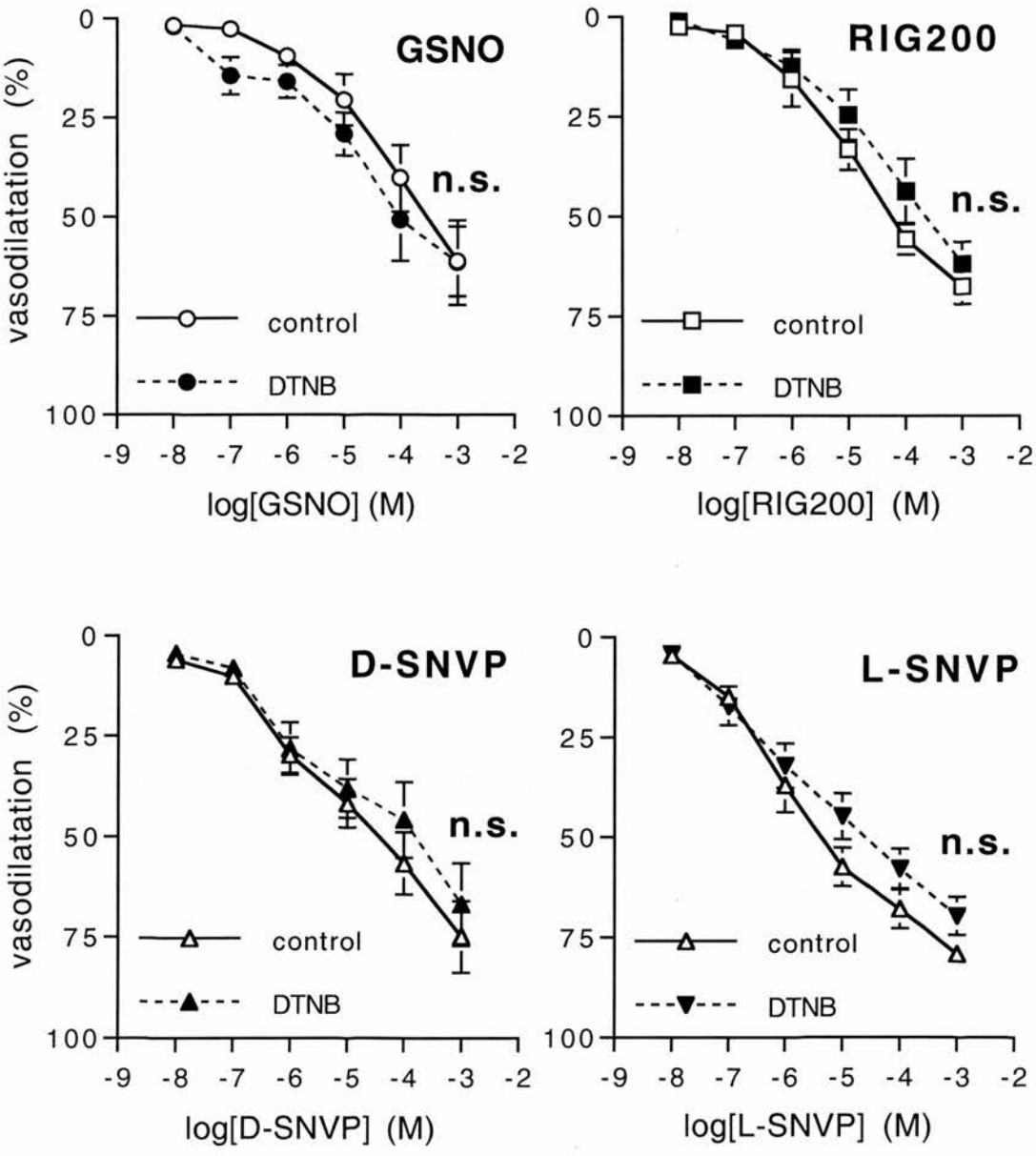


Figure 4.3.1 Effect of DTNB (100 μM) on vasodilator responses to NO donors (10 μl); (a) GSNO, (b) RIG200, (c) D-SNVP and (d) L-SNVP. Points shown are means ± S.E.M. (n=6-7).

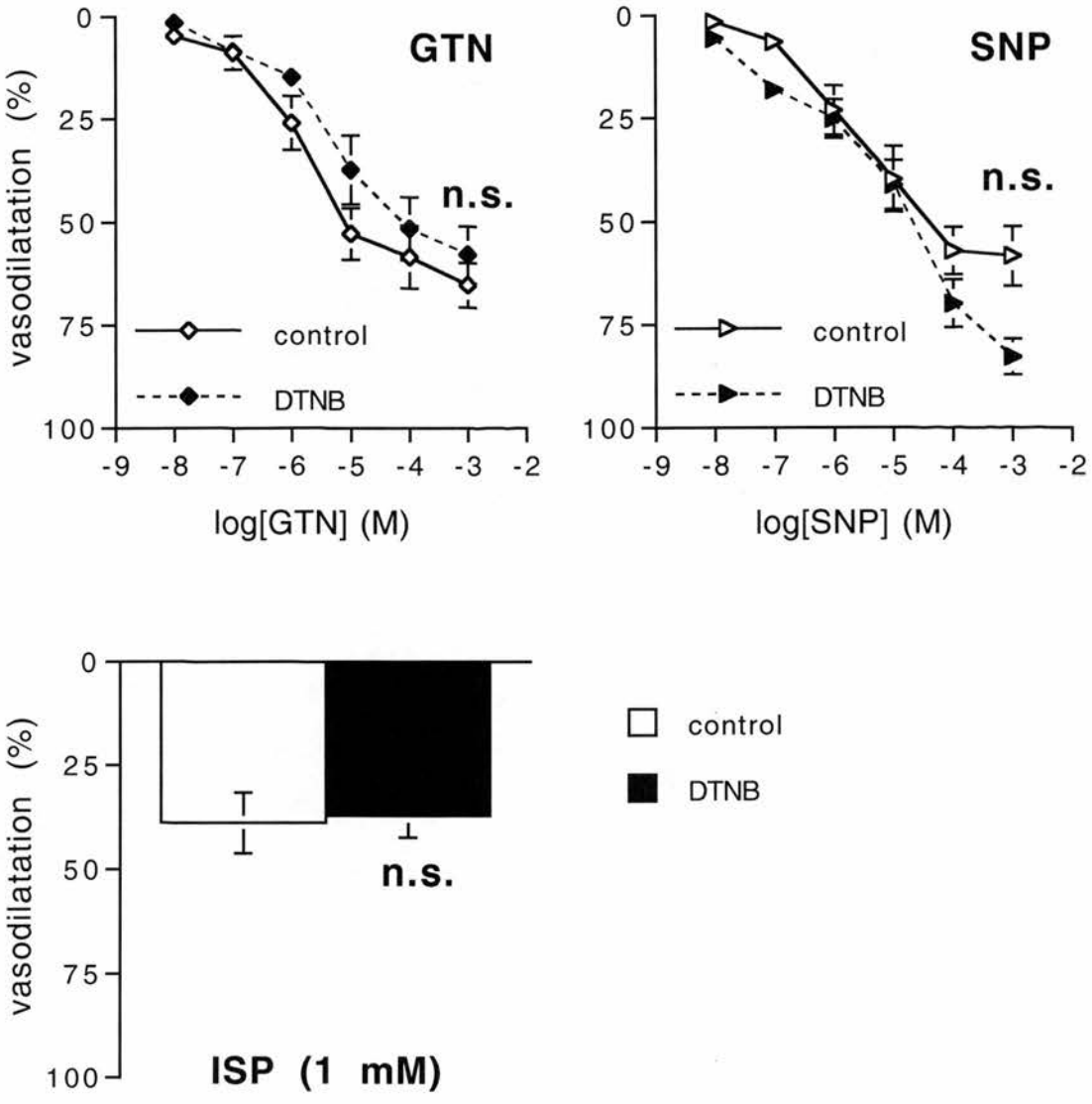


Figure 4.3.2 Effect of DTNB (100 μM) on vasodilator responses to NO donors (10 μl); (a) GTN, (b) SNP and (c) ISP. Points shown are means ± S.E.M. (n=6 for all).

4.3.5 The effect of BSO on responses to vasodilators

20 h of BSO perfusion (100 μM) attenuated the response to microinjections of all NO donors (P<0.005; n=6-9), with the exception of D-SNVP where the degree of inhibition did not reach statistical significance (P=0.08; n=7; Fig 4.4). BSO also attenuated the response to microinjection (10^{-3} M) of ISP from 43.0±4.4% to 28.4±3.5% (P=0.018; paired t-test; n=13; Fig 4.4.2). Vasodilator responses to ISP were inhibited to a lesser extent than vasodilatation to NO donors, where a control response of ~43% was reduced to between 15-25% for all NO donors, except for GTN which was reduced to ~12%.

4.3.6 The effect of EA on responses to vasodilators

EA (100 μM) produced a significant attenuation of responses to all the NO donors tested (P<0.003 for all; n=6-11; Fig 4.5). EA also significantly attenuated the response to ISP (10^{-3} M), reducing vasodilatation from 45.9±5.4 to 18.0±2.5% (P=0.007; paired t-test; n=6: Fig 4.5.2). GSNO, GTN and SNP in particular, showed the greatest attenuation, with a control response of ~45% reduced to ~10%, compared to RIG200, D-SNVP, L-SNVP and ISP where a control response of ~45% was reduced to ~20-25%. In all cases, the degree of inhibition caused by EA was greater than that of BSO, although this difference was only statistical significance for SNP and GSNO (P=0.04 for both; 2-way-unrelated ANOVA; n=6-11).

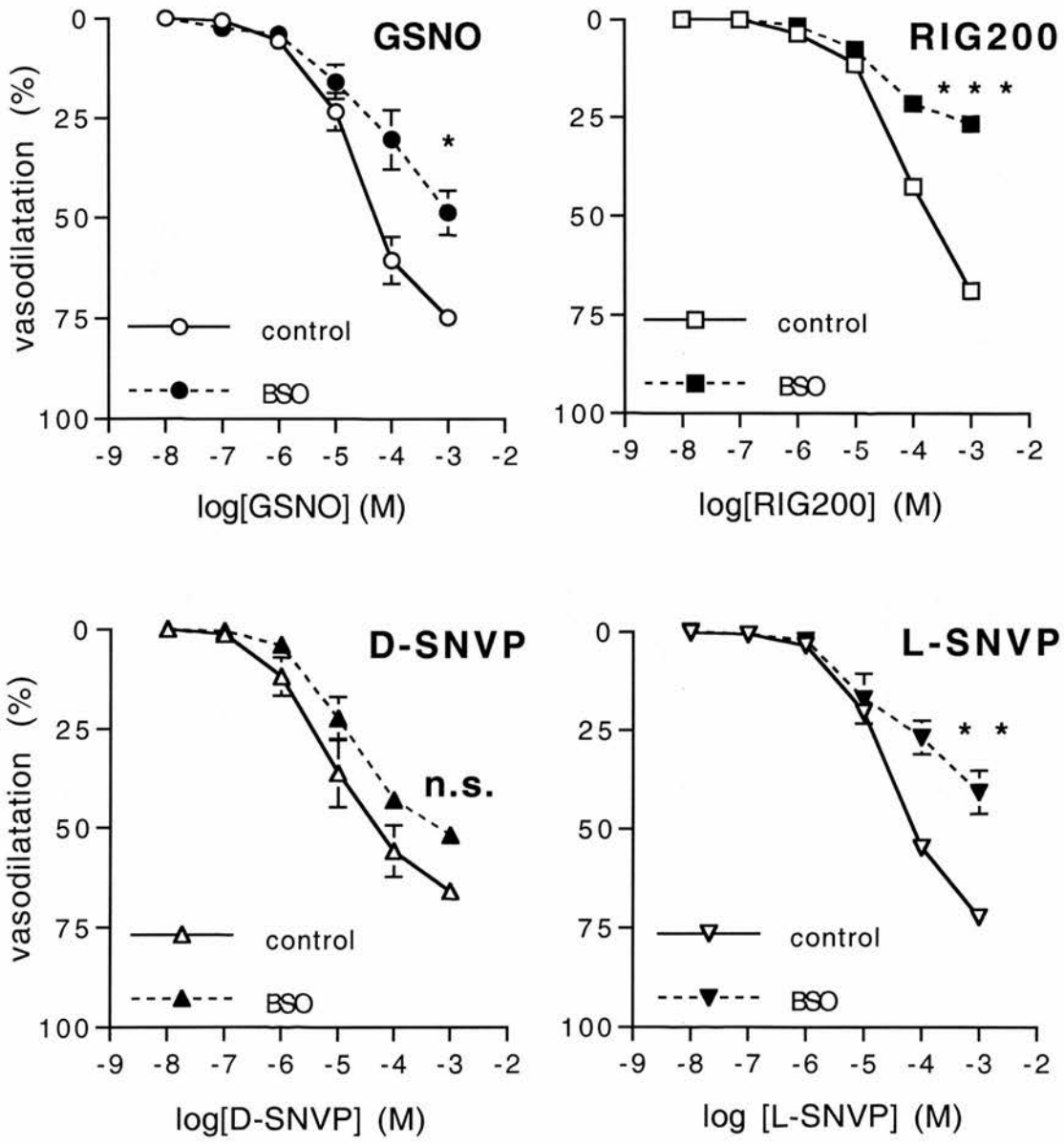


Figure 4.4.1 Effect of BSO (100 μM) on vasodilator responses to NO donors (10 μl); (a) GSNO, (b) RIG200, (c) D-SNVP and (d) L-SNVP. Points shown are means ± S.E.M. (n=6-7).

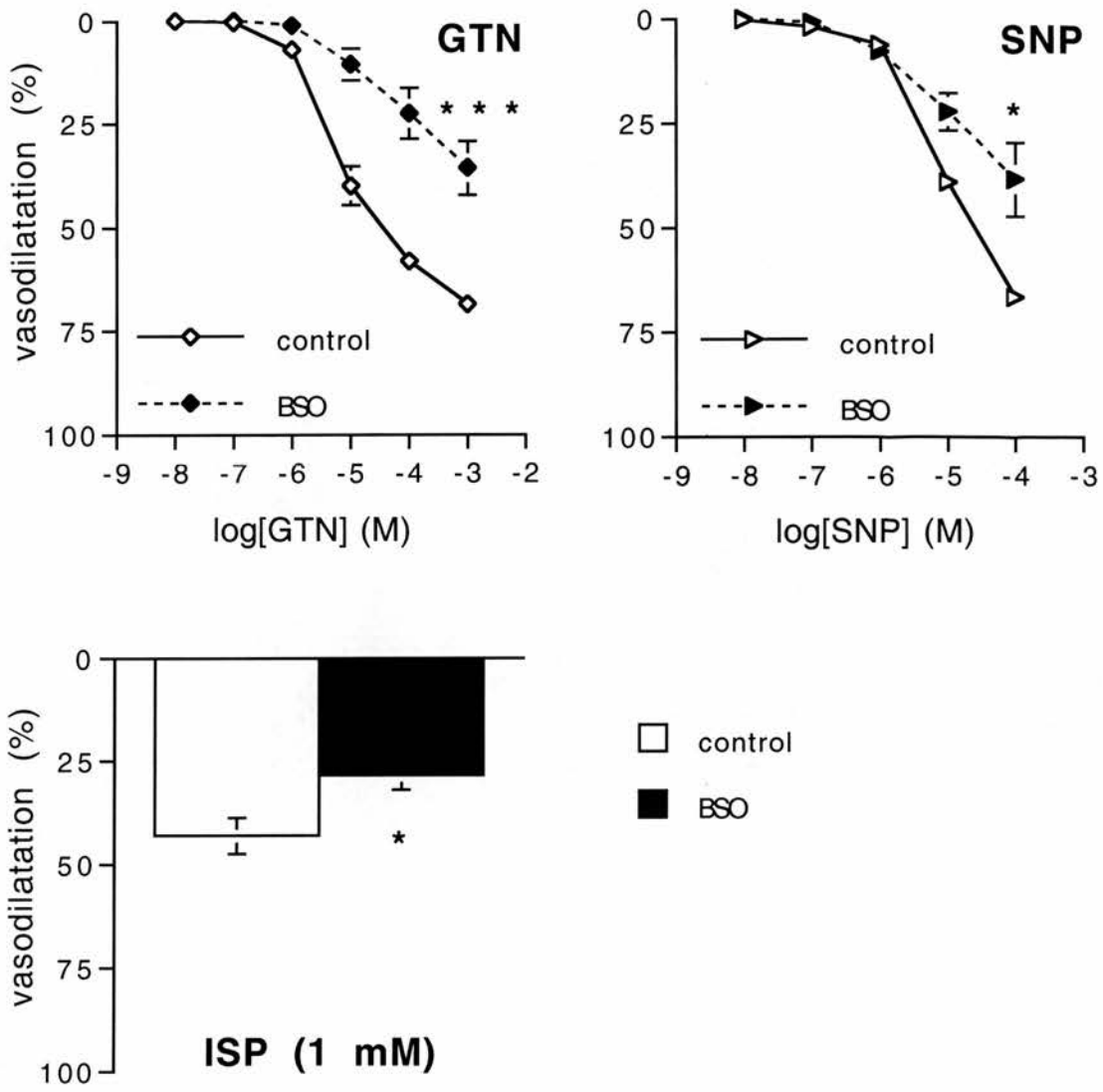


Figure 4.4.2 Effect of BSO (100 μM) on vasodilator responses to NO donors (10 μl); (a) GTN, (b) SNP and (c) ISP. Points shown are means ± S.E.M. (n=6-13).

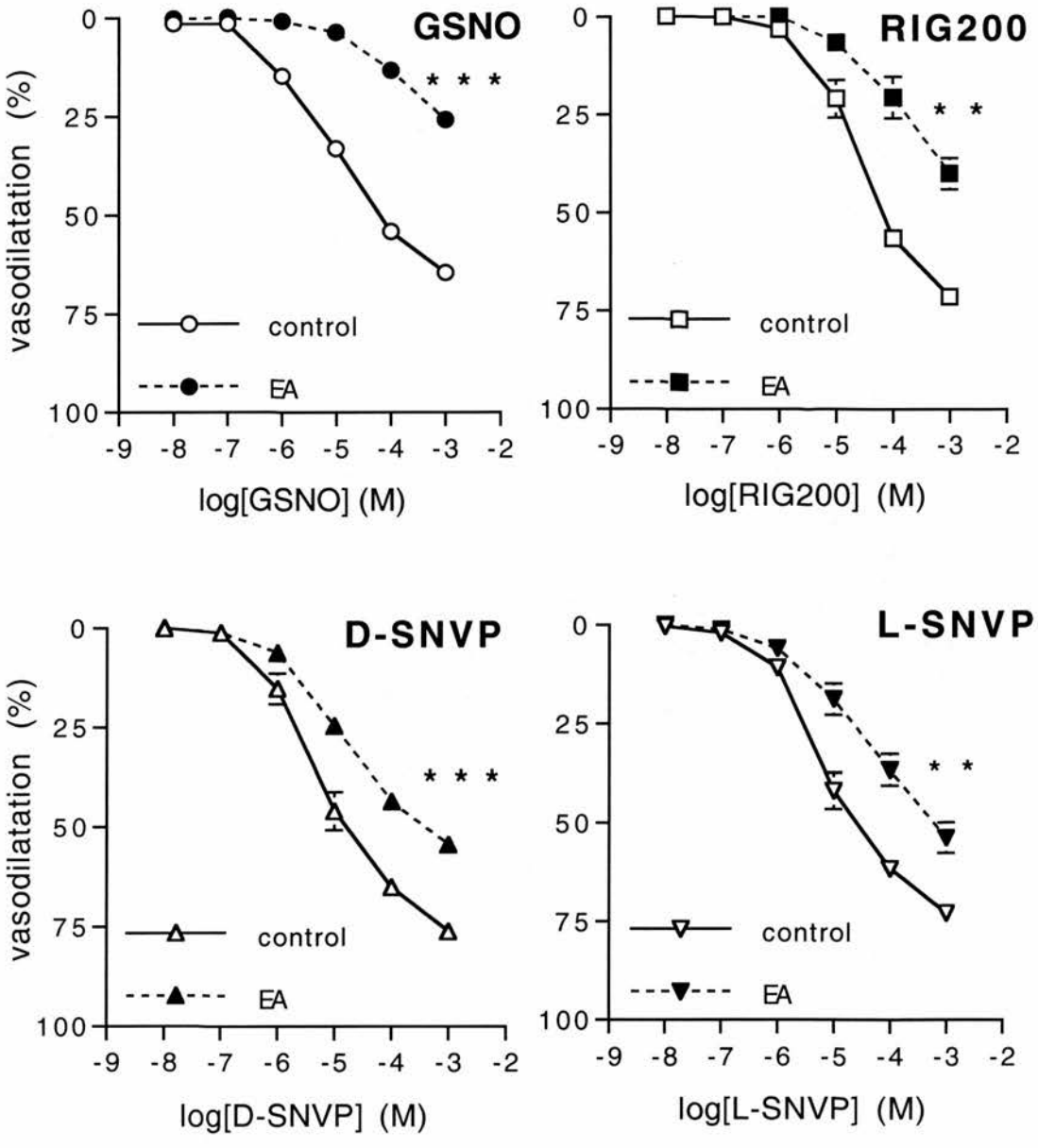


Figure 4.5.1 Effect of EA (100 μM) on vasodilator responses to NO donors (10 μl); (a) GSNO, (b) RIG200, (c) D-SNVP and (d) L-SNVP. Points shown are means ± S.E.M. (n=6-11).

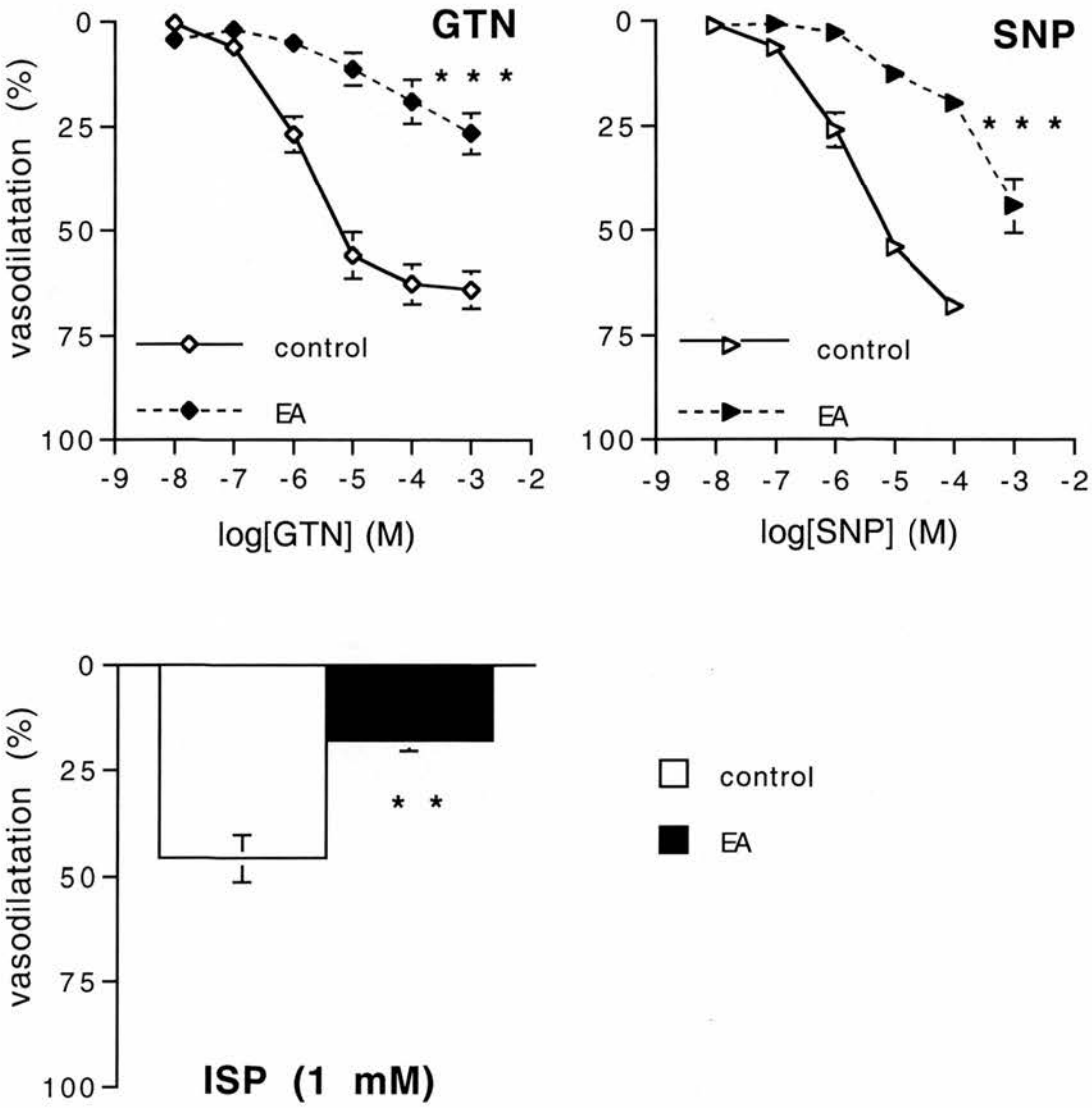


Figure 4.5.2 Effect of EA (100 μM) on vasodilator responses to NO donors (10 μl); (a) GTN, (b) SNP and (c) ISP. Points shown are means S.E.M. (n=6-11).

4.4 DISCUSSION

At face value, the results show that intracellular, but not extracellular, thiol groups modulate the activity of NO donors. Furthermore, it is apparent that intracellular GSH is a highly important reservoir of anti-oxidant thiols and that protein thiol groups are important for both the vasodilator activity of NO donors and compounds that act independently of the NO:sGC pathway.

4.4.1 Vasodilator potency of NO donors

Given the different activation mechanisms of NO donors, their ability to cause vasodilatation is strikingly similar. Interestingly, there was a significant difference between the D- and L-stereoisomers of SNVP. Other investigators have demonstrated that stereoisomers of both endogenous (SNOC; Davisson *et al.*, 1996) and exogenous (S-nitrosopenicillamine; Travis *et al.*, 1997; Travis *et al.*, 1996) S-nitrosothiols cause different haemodynamic effects *in vivo*. The authors suggest that this may reflect the ability of stereoisomers to bind to an endogenous S-nitrosothiol receptor. However, these effects were masked by the use of anaesthetics (Travis *et al.*, 1997) and appear to be mediated through the central nervous system (Davisson *et al.*, 1997) rather than the vascular system. In the current study, the difference in the vasodilator potency of D- and L-SNVP was modest and unlikely to be physiologically relevant. Together with the findings of Kawamoto *et al*, who, despite extensive experimentation, were unable to provide evidence for a nitrate receptor (Kawamoto *et al.*, 1988), these results suggest that the primary mechanism of NO donors is not through binding to a receptor, but through biotransformation to NO. Stereoselectivity of NO donors is, therefore, more likely to be due to differences in binding to enzymes that metabolise these drugs.

4.4.2 The role of cell surface thiols

DTNB, or Ellman's reagent, blocks reduced SH groups by forming a mixed disulphide with the thiol and is often used to estimate the level of available SH groups (Ellman, 1959; Gergel & Cederbaum, 1997). Because DTNB is a large molecule, it does not cross the cell membrane (Tang & Aizenman, 1993) and subsequently it can be used as a specific inhibitor of cell surface thiols, leaving intracellular thiols intact. As expected, DTNB pretreatment had no effect on the vasodilator activity of either GTN or SNP, in accordance with their intracellular metabolism (Bennett *et al.*, 1989; Chung & Fung, 1990; Schror *et al.*, 1991; see Chapter 3). However, DTNB also had no effect on any of the S-nitrosothiols, regardless of their lipophilicity. The lack of effect of DTNB suggests that transnitrosation of cell-surface thiols is not a pre-requisite for S-nitrosothiol-mediated vasodilatation.

Evidence has been presented to show that proteins containing a reduced SH group are involved in the transfer of NO across cell membranes. In red blood cells, anion exchange protein 1 (AE1) has been implicated in the export of NO from intracellular S-nitrosated-Hb (SNO-Hb; Pawloski *et al.*, 2001). It was proposed that SNO-Hb transnitrosates a thiol group of AE1, which then mediates the passage of NO across the red blood cell membrane to plasma thiols. This mechanism could act to protect SNO-Hb-derived NO from direct scavenging by the haem group of Hb. More recently, attention has focused on cell surface protein disulphide isomerase (csPDI), a protein in the cell membrane which is involved in the maintenance of the redox status of cell surface thiols (Jiang *et al.*, 1999; Zai *et al.*, 1999). It was demonstrated that S-nitrosothiols transnitrosated one of the SH groups of csPDI, and that this process was required for stimulation of sGC (Zai *et al.*, 1999). More recently, another group suggested that the thiol groups of csPDI cleave NO from S-nitrosothiols without the need for S-nitrosation of PDI (Ramachandran *et al.*, 2001). NO is taken up into the lipid regions of the cell membrane, where it reacts with oxygen, and the resulting nitrogen oxides (e.g. NO_2, N_2O_3, N_2O_4) then transnitrosate intracellular thiols.

The results of the AE1 and csPDI experiments are convincing, but both proposals require a reduced SH group to be available. This begs the question as to whether the present results using DTNB are misleading. There are two potential limitations in the use of DTNB as an extracellular thiol inhibitor. Firstly, concentrations of DTNB may not be sufficient to block all available surface thiols, especially in the presence of SH-replenishing antioxidant systems, such as csPDI. However, the concentrations of DTNB could not be increased due to the cytotoxicity of this compound. Also, the large size of DTNB may actually be a limitation. Reduced sulphydryl groups of proteins can be buried within the molecule, in hydrophobic clefts (Leeuwenkamp *et al.*, 1986). Therefore, the bulky structure of DTNB may restrict access to crucial SH groups that can be regulated by smaller molecules such as nitrogen oxides. Indeed, Jiang et al. demonstrate that DTNB was not capable of oxidising all of the available SH groups of csPDI (Jiang *et al.*, 1999). Experiments with smaller extracellular thiol alkylators are now needed to clarify the role of csPDI, AE1 and other SH-containing cell surface proteins, as well as test the hypothesis that sGC-independent actions of NO donors are mediated by S-nitrosation of cell surface SH groups (see Chapter 3).

4.4.3 The role of intracellular thiols

GSH is the most common intracellular thiol in VSMCs (Gruetter & Lemke, 1985; Boesgaard *et al.*, 1993; Haj-yehia & Benet, 1996). GSH levels are maintained through the recycling of oxidised GSSG by GSH reductase and the *de novo* synthesis of GSH by γ-glutamylcysteine synthase (Griffith, 1999; Sies, 1999). BSO specifically inhibits γ-glutamylcysteine synthase and prolonged incubation (20 h) depletes intracellular GSH as it is used in enzymatic processes, transported out of the cell and metabolised to Cys-glycine and glutamate (Meister, 1983; Griffith, 1999; Sies, 1999). The loss of an essential thiol such as GSH, will undoubtedly compromise antioxidant defence in

VSMCs, resulting in the accumulation of oxygen free radicals from normal cellular metabolism (Meister, 1994). Depletion of GSH could affect the vasodilator actions of NO donors in several ways; by influencing their metabolism, by the loss of protection against NO-scavenging superoxide or by regulation of sGC activity through modulation of the redox state of the SH groups in its catalytic site (Kamisaki *et al.*, 1986).

BSO attenuated the vasodilator actions of the NO donors to variable extents. The S-nitrosothiols tended to be less susceptible to GSH depletion. Loss of thiols may inhibit the release of NO from S-nitrosothiols by preventing the reduction of Cu(II) to Cu(I) (Dicks *et al.*, 1996; Singh *et al.*, 1996; Al-Sa'doni *et al.*, 1997; Holmes & Williams, 1998) or by limiting the availability of thiols that undergo transnitrosation reactions (Park, 1988; Askew *et al.*, 1995). Subsequently, the relatively stable S-nitrosothiols used in this study would not be able to release NO to stimulate sGC. However, both these mechanisms could still occur via the residual thiols remaining after BSO pretreatment. Additionally, the transport of NO as an S-nitrosothiol may act as a mechanism to protect NO from scavenging by superoxide (Upchurch *et al.*, 1995; Gaston, 1999; Moore & Mani, 2002). SNP was also relatively resistant to GSH depletion. Thiols have been shown to chemically reduce SNP, but it is unclear if this process is required for NO release (Butler & Megson, 2002).

Both *in vivo* (Boesgaard *et al.*, 1993), and *in vitro* (De Man *et al.*, 1996), administration of BSO has been demonstrated to inhibit the vasodilator response of GTN. Responses to GTN were found to be inhibited by BSO in the current study, to a much greater extent than the other NO donors. Previously, the inhibitory effects of thiol depletion on GTN contributed to the hypothesis that depletion of free thiols was the underlying cause of nitrate tolerance, by preventing thiol-mediated biotransformation of GTN (Needleman *et al.*, 1973). However, vasodilatation to ISP is also attenuated in vessels that have been depleted of thiols (discussed below), but remains fully active in vessels that have been made tolerant to nitrates (Needleman &

Johnson, 1973). Although reduced biotransformation of GTN may still be the cause of tolerance, it is now generally accepted that free thiols alone are not responsible for release of NO from GTN under physiological conditions (Megson, 2000; see Chapter 5).

ISP was also inhibited by BSO pretreatment, albeit to a lesser extent than the NO donors. Cell death was not the cause of this inhibition as viability was confirmed by the restoration of perfusion pressure in response to PE after the PE-free period during incubation with BSO. Indeed, GSH levels need to be depleted by over 90% before there is a loss of cell viability (Dethmers & Meister, 1981; Rossi *et al.*, 1986) and this is unlikely to occur with less than 24 h BSO incubation, unless additional stresses are applied (Moore *et al.*, 1987; Griffith, 1999).

The non-specific inhibition of NO:sGC-independent vasodilators following depletion of intracellular thiols is not necessarily surprising. As shown in Chapter 3, an increase in oxidative stress following inhibition of the antioxidant protection of SOD led to a small reduction in ISP-induced vasodilatation. Reduced SH groups are present in many Cys-containing proteins and if GSH is sufficiently compromised to allow oxygen free radicals to accumulate, then non-specific oxidation of these SH groups will occur.

To investigate this further, the thiol-alkylator, EA, was used to induce non-specific alkylation of both free thiols and the SH groups of proteins (Komorn & Cafruny, 1965). The non-specific nature of EA is highlighted by the large increase in pressure on perfusion of EA, despite the absence of endothelium-derived NO. EA produced a large attenuation of the responses to all vasodilators particularly GTN and SNP, consistent with earlier findings (Needleman *et al.*, 1973; Lau & Benet, 1992). EA also decreased the vasodilator response to ISP, a compound that acts through AC rather than sGC, confirming the findings of Needleman *et al* (Needleman *et al.*, 1973). Vasodilatation in response to ISP could have been inhibited by the oxidation of sulphydryl groups of AC (Guillon *et al.*, 1981), the receptor-AC coupling protein

(Mukherjee & Mukherjee, 1981; Suen *et al.*, 1982) or at a common site downstream of AC activation (Needleman *et al.*, 1973), such as the SH groups of myosin (Kubberod *et al.*, 1974; Stamler *et al.*, 1992).

Although it would be unproductive to speculate further on the exact mechanism of thiol depletion on the widespread inhibitory effect to vasodilators, this study highlights that caution should be exercised when interpreting the results from experiments using thiol-depletors. The lack of specificity of agents that deplete reduced thiols is not unique to vascular tissue and has been noted in other biological models, particularly platelet aggregation (Stamler & Slivka, 1996).

4.4.4 Summary

Despite the fact that DTNB is commonly used to block extracellular thiol groups, it has yet to be established that DTNB can sufficiently block crucial SH groups on cell surface proteins. Subsequently, the results of this study only allow speculation of the role of cell surface thiols in the decomposition of NO donors and the transduction of NO across the plasma membrane. However, this study clearly demonstrates that intracellular thiols are required for the vasodilator action of all NO donors, particularly GTN. Care should be taken in the interpretation of results from the use of thiol-depletors in experiments that investigate NO physiology, as thiol availability is also important for the effects of drugs that do not act through the NO:sGC pathway.

Chapter 5

Novel S-nitrosothiols do not engender vascular tolerance and remain effective in glyceryl trinitrate-tolerant rat femoral arteries

5. NOVEL S-NITROSOTHIOLS DO NOT ENGENDER VASCULAR TOLERANCE AND REMAIN EFFECTIVE IN GLYCERYL TRINITRATE-TOLERANT RAT FEMORAL ARTERIES

5.1 INTRODUCTION

Organic nitrates, such as GTN, are currently used for angina, and the symptomatic relief in severe cardiac ischaemia, myocardial infarction and heart failure (Abrams, 1985). However, the therapeutic use of nitrates is limited by the development of tolerance, where a diminished effectiveness of these drugs is seen within 24 h of continuous therapy (Parker & Fung, 1984). Tolerance can be demonstrated *in vitro*, suggesting direct impairment of a vascular mechanism, such as inefficient biotransformation of GTN (Brien *et al.*, 1986; Bennett *et al.*, 1989; Slack *et al.*, 1989; Feelisch & Kelm, 1991), desensitization of the target enzyme, guanylate cyclase (Needleman & Johnson, 1973; Axelsson & Andersson, 1983; Waldman *et al.*, 1986) or upregulation of cGMP-metabolising phosphodiesterases (Axelsson & Andersson, 1983; Kim *et al.*, 2001). Recently, it has been demonstrated that tolerance induction *in vivo* is associated with elevated superoxide production due to enhanced activity of endothelial NAD(P)H oxidases (Munzel *et al.*, 1995; Munzel *et al.*, 1996), expression of a dysfunctional form of eNOS (Kaesemeyer *et al.*, 2000; Munzel *et al.*, 2000), and reduced levels of vascular Cu/Zn-SOD (Munzel *et al.*, 1999). Superoxide reacts with NO, forming cytotoxic products, such as peroxynitrite, and reducing NO

bioavailability (White *et al.*, 1994). Weight is added to this arguement by clinical studies demonstrating that tolerance can be prevented or partially reversed with anti-oxidant vitamins (Watanabe *et al.*, 1997; Bassenge *et al.*, 1998; Watanabe *et al.*, 1998; Bassenge *et al.*, 2001).

The induction of tolerance appears to be unique to the organic nitrates, as other classes of NO donors, such as SNP (Kieth *et al.*, 1982; Kowaluk *et al.*, 1987; Hinz & Schroder, 1998; Sage *et al.*, 2000; Minamiyama *et al.*, 2001), SIN-1 (Sutsch *et al.*, 1989; Rudolph & Dirschinger, 1991; Hinz & Schroder, 1998) and the NONOates (Hinz & Schroder, 1998) do not show susceptibility to tolerance. This may be because they lack the strict metabolic requirements of the organic nitrates and subsequently release NO more readily. S-Nitrosothiols, in particular, do not appear to require biotransformation to activate guanylate cyclase (Ignarro *et al.*, 1981), suggesting that they may not be susceptible to tolerance. Indeed, a number of S-nitrosothiols, including SNAP, have been shown to develop little or no self-tolerance with continuous treatment, and also to remain effective in GTN-tolerant vessels *in vitro* (Kowaluk *et al.*, 1987; Kowaluk & Fung, 1990; Matsumoto *et al.*, 1995; Hanspal *et al.*, 2002) and *in vivo* (Bauer & Fung, 1991; Shaffer *et al.*, 1992).

At present it is unknown if the structural alterations made to SNAP to improve stability and lipophilicity affect its tolerance profile. In order for the benefits of SNAP analogues such as RIG200 and SNVP to be maximally exploited therapeutically, it is eseential that these novel compounds do not engender tolerance with continued use. This study uses a model of nitrate tolerance in isolated rat femoral arteries to test the hypothesis that RIG200 and D-SNVP do not induce vascular tolerance or show cross-tolerance with GTN.

5.2 METHODS

5.2.1 Preparation

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (250-350 g; n=93) in a perfusion system. The vessels perfused (0.6 ml min^{-1}) and superfused (1 ml min^{-1}) with fresh oxygenated (95% O_2, 5% CO_2) Krebs buffer solution. Vessel tone was measured by monitoring perfusion pressure with a differential pressure transducer. All experiments were carried out in a darkened laboratory (see Section 2.3.2).

5.2.2 Experimental protocols

Vessels were preconstricted with phenylephrine (PE; 2-10 μM) in the presence of supramaximal concentrations of the NO synthase inhibitor L-NAME (20 μM; see Section 2.3.2).

5.2.2.1 *Induction of tolerance*

Vessels were perfused with equivalent concentrations of NO donor (10 μM), or Krebs buffer as a control, and perfusion pressure was monitored for 2 h. PE was then removed from the internal perfusate and the perfusion rate lowered to 0.1 ml min^{-1} overnight, at 25°C, to optimise vessel survival. At t=20 h, the original PE-containing solution was re-perfused at the original flow rate (0.6 ml min^{-1}), at 37°C (Fig 5.1).

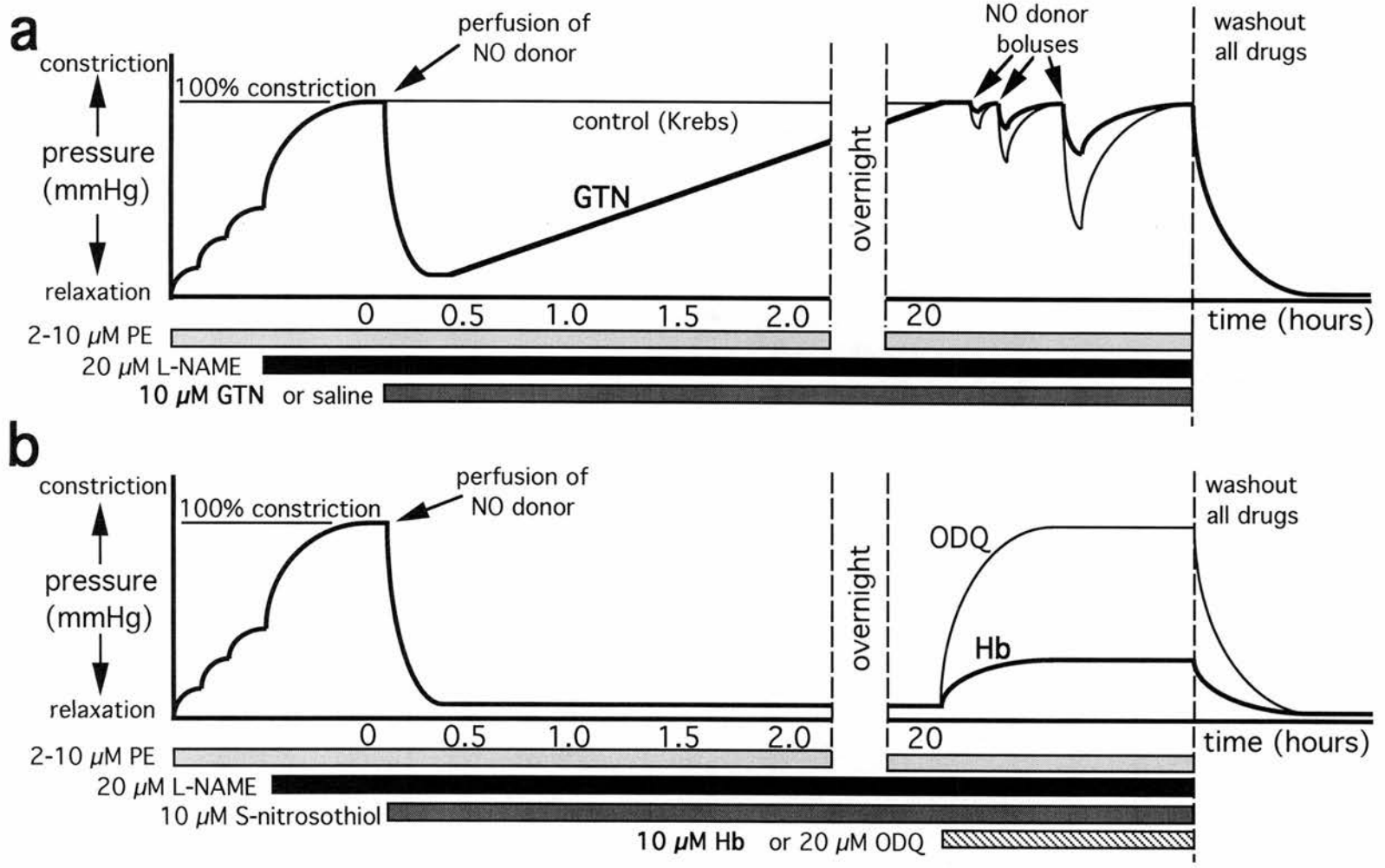


Figure 5.1 Schematic diagram of the experimental protocols used in this study. Cross-tolerance to NO donors could only be investigated if perfusion pressure was restored on perfusion of NO donors (GTN, bold line; or control, thin line; a). Note that perfusion of NO donors was continued until the end of the experiment and was present throughout boluses of NO donors (a) and co-perfusion of Hb (bold line) or ODQ (thin line) (b).

5.2.2.2 Cross-tolerance

In GTN-treated and control vessels (t=20 h), bolus injections of increasing concentrations of NO donor (10 µl; 10^{-8}-10^{-3} M) were made sequentially into the perfusate. Responses were deemed to have recovered once pressure was maintained for more than 2.5 min, at which time the next concentration was injected (Fig 5.1a).

5.2.2.3 *Washout of NO donor*

To confirm the viability of vessels that did not develop tone on exposure to PE following 20 h of S-nitrosothiol perfusion, the S-nitrosothiol was washed out and the time taken for maximum pressure to be restored was measured.

5.2.2.4 Nature of NO donor vasodilatation

In S-nitrosothiol-treated vessels at t=20 h, the NO scavenger, Hb (10 µM; (Martin *et al.*, 1985) was added to the internal perfusate, and subsequently to the superfusate to allow Hb to infiltrate the vascular smooth muscle, as it has been shown previously that the endothelium may act as a barrier to Hb (Foley *et al.*, 1993). Hb-induced responses were deemed complete after pressure was maintained for 5 min. A supramaximal concentration (20 µM) of the soluble guanylate cyclase inhibitor, ODQ (Garthwaite *et al.*, 1995), was added to the internal perfusate and rapidly washed out once pressure had reached plateau (Fig 5.1b).

5.2.3 Analysis of Results

Vasodilator response amplitude is the decrease in pressure, expressed as a % of preconstriction pressure existing before the application of each drug concentration (%

pressure change; positive values represent vasodilatation, where 100% represents maximum vasodilatation). Mean values are given ± S.E.M..

P-values in the text were obtained by two-factor, unrelated analysis of variance (ANOVA). Paired and unpaired, two-tailed Student's *t*-tests were all two-tailed

5.3 RESULTS

5.3.1 Vessel preconstriction

Vessels were preconstricted with phenylephrine (6.7±0.3 μM) to give pressures of ~50 mmHg (49±3 mmHg; n=60). L-NAME (20 μM) led to a 151±13% increase of pre-existing PE-induced pressure to generate a final pressure of 110±5 mmHg; (n=60).

5.3.2 Vasodilator responses to continuous NO donor perfusion

Perfusion of GTN (10 μM) caused an initial vasodilatation of 72±3% (n=33). Pressure gradually recovered to 35±10% vasodilatation remaining at t=2 h (Fig 5.2a and 5.3). After overnight incubation with GTN (t=20 h), pressure was not significantly different from control (-10±10%; P=0.64; unpaired t-test; n=45).

Perfusion of supramaximal concentrations of S-nitrosothiols (10 μM) produced greater vasodilatation (91±2, 93±1, 84±3% for GSNO, RIG200 & D-SNVP respectively; n=12-15), which were maintained throughout the 20 h period of perfusion (Fig 5.2a and 5.3).

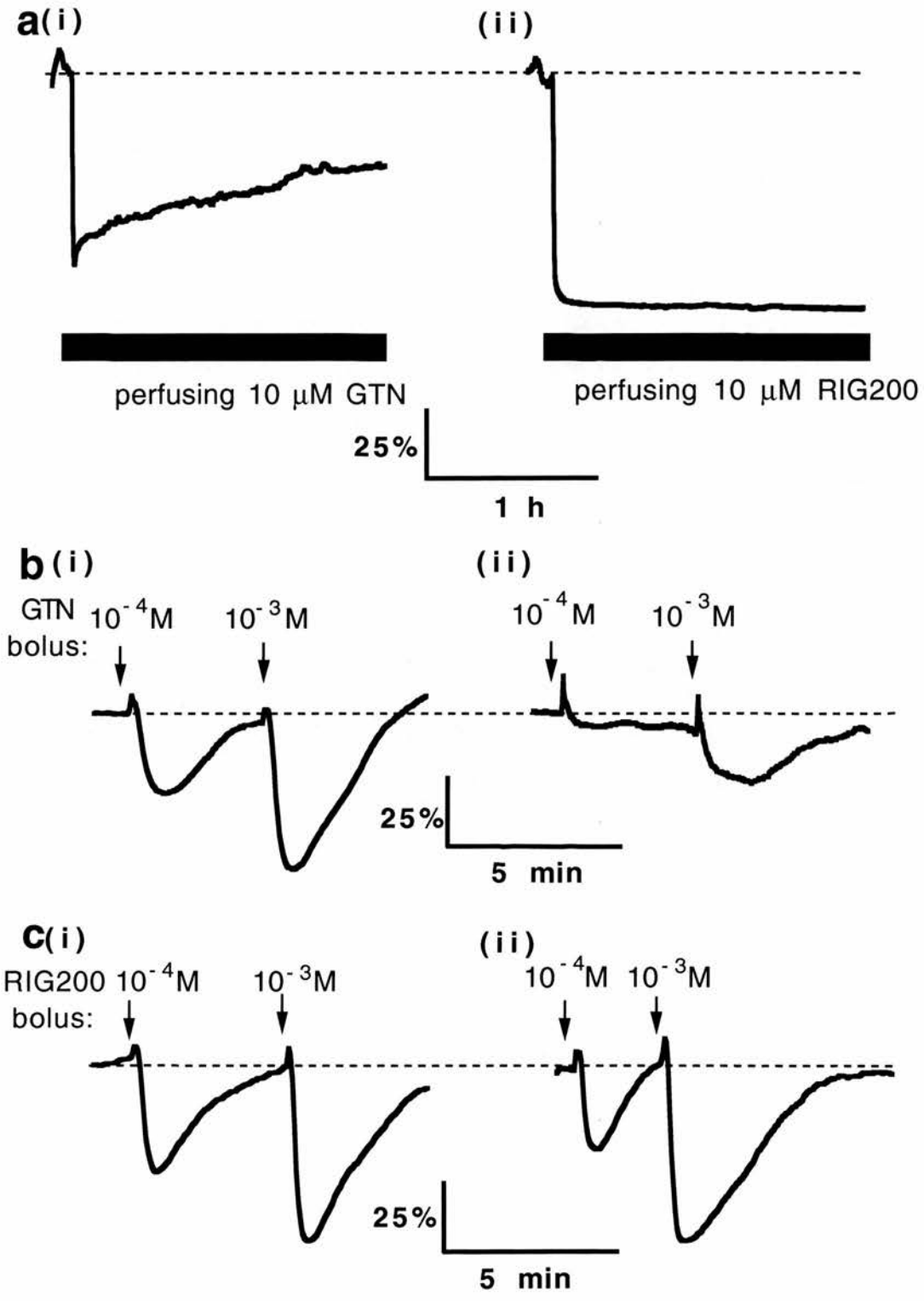


Figure 5.2 Pressure recordings showing vasodilator responses. a) continuous perfusion (10 μM) of (i) GTN or (ii) RIG200. b) responses to sequential micro-injections of GTN (10 μl; 10^{-4}, 10^{-3} M) into the perfusate of (i) control and (ii) GTN-tolerant vessels. c) responses to sequential micro-injections of RIG200 (10 μl; 10^{-4}, 10^{-3} M) into the perfusate of (i) control and (ii) GTN-tolerant vessels.

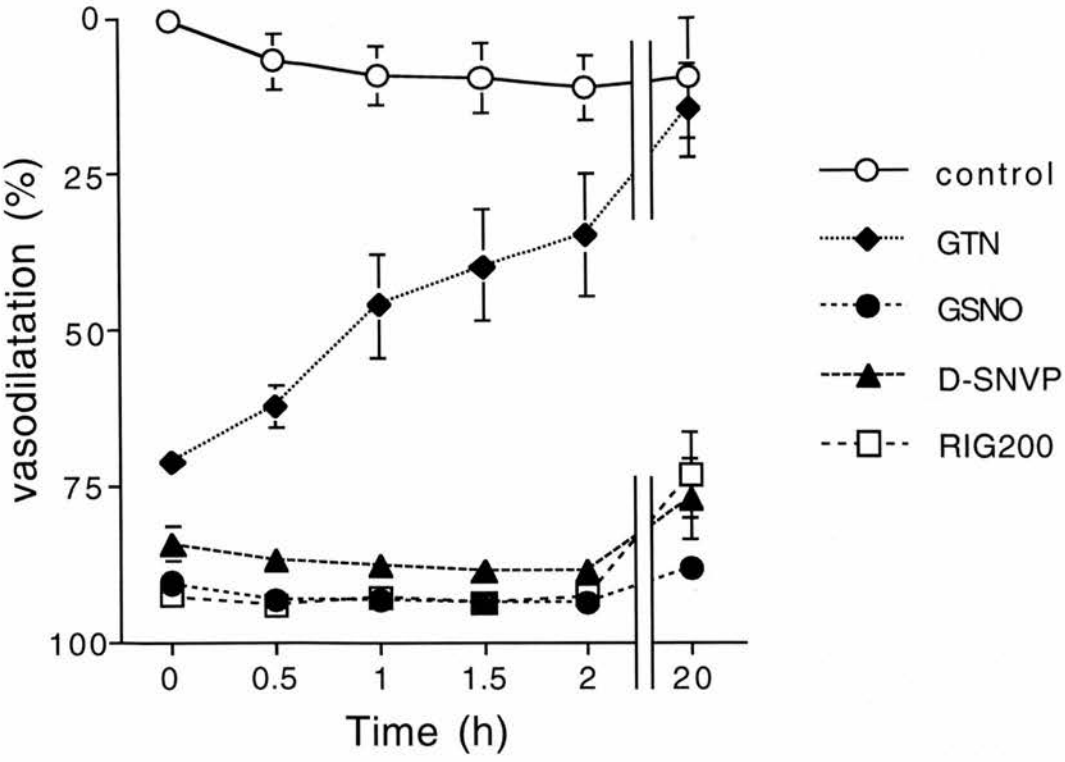


Figure 5.3 Effect of perfusing NO donors (10 μM) on perfusion pressure in preconstricted rat femoral arteries. 100% vasodilatation represents abolition of tone, with NO donor perfusion beginning at t=0 h. Points shown are means with vertical lines indicating S.E.M. (n=45, 33, 15, 12, 12 for Control, GTN, GSNO, RIG200 & D-SNVP respectively).

5.3.3 Vasodilator responses to bolus injections of NO donors in control and GTN-tolerant vessels

Bolus injections of GTN (10 μl; 10^{-8}-10^{-3} M) produced transient vasodilatations which recovered within 5 min. In vessels perfused overnight in the absence of GTN (control), the highest concentration of GTN tested (10^{-3} M) produced a vasodilatation of 65 ±7% (n=6). In vessels perfused with GTN for 20 h (GTN-tolerant vessels) the responses to bolus GTN were markedly attenuated (19±4 %; $P<0.001$; n=7; Fig 5.2b and 5.4a).

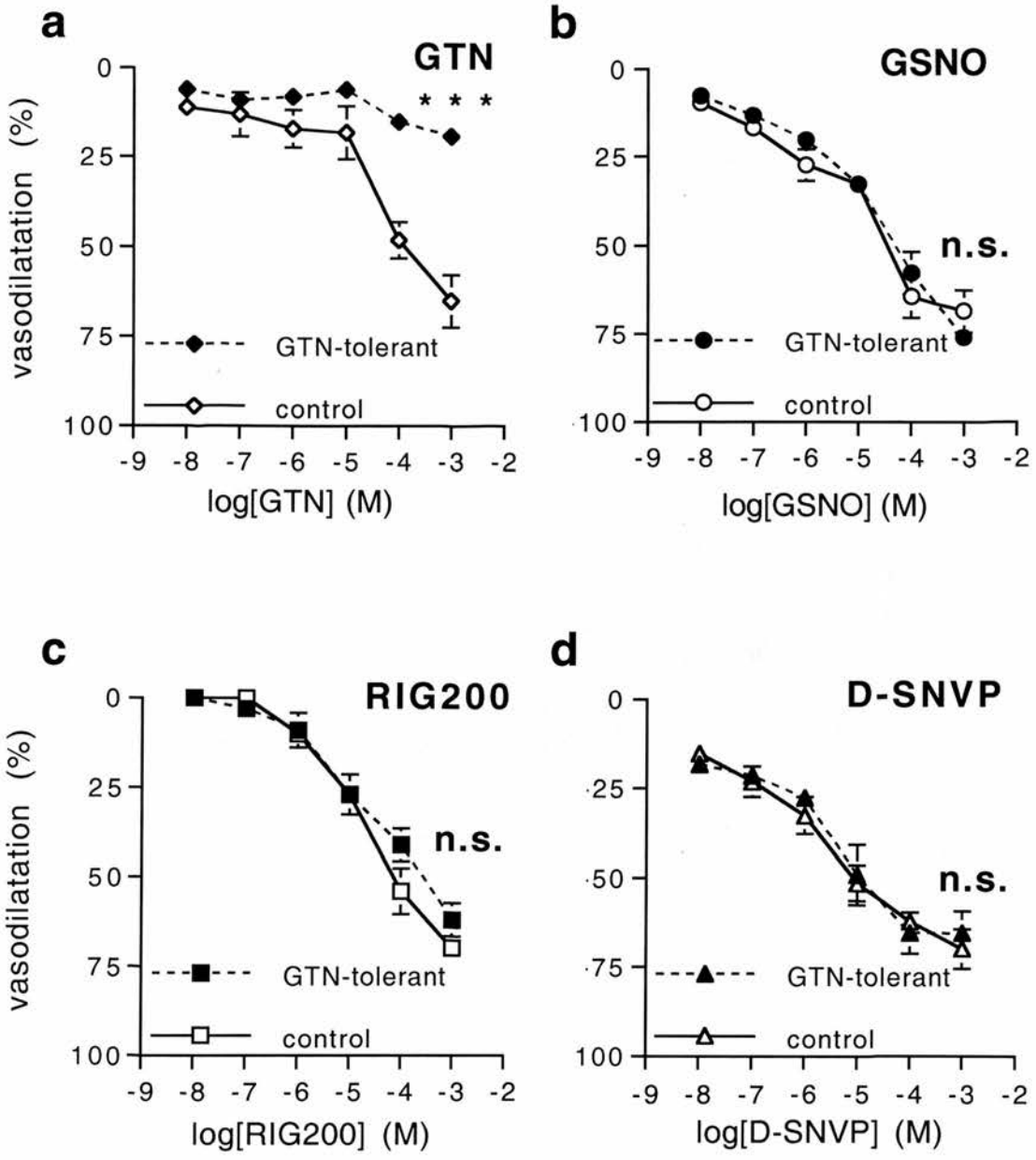


Figure 5.4 Log concentration response curves showing the vasodilator effect (% preconstriction pressure) of bolus microinjections (10 µl) of a) GTN, b) GSNO, c) RIG200, d) D-SNVP in control (open symbols) and GTN-tolerant (filled symbols) vessels. Points shown are means with vertical lines indicating S.E.M. (n=6-9).

Equivalent injections of S-nitrosothiols also produced transient vasodilatations of a similar amplitude to GTN (69±6, 70±3 & 70±6% for GSNO, RIG200 and D-SNVP respectively; Fig 5.2b and 5.4b-d). However, the concentration-response curves for the S-nitrosothiols in GTN-tolerant vessels were not significantly different from those in control vessels (P>0.21; n=6-9).

5.3.4 Washout of S-nitrosothiols

At t=20 h, the internal perfusate was replaced with Krebs solution containing PE and L-NAME, but without NO donor. On washout of GSNO, RIG200 or D-SNVP, pressure recovered to levels that were not significantly different from the preconstriction pressure before the perfusion of NO donor (P=0.15; paired t-test; n=18; Fig 5.5). Pressure rapidly recovered in 5.5±0.9, 6.5±0.8 and 11.1±4.5 min, respectively (n=6 for all).

5.3.5 Reversal of S-nitrosothiol vasodilatation with Hb and ODQ

Following perfusion of GSNO for 20 h, addition of Hb (10 μM) to the internal perfusate caused a significant increase in pressure of ~40% (P=0.005, paired$t$-test; n=6). Hb had no effect on the vasodilatation produced by RIG200 or D-SNVP (P>0.15 for both; n=6; Fig 5.5). Addition of Hb (10 μM) to the external perfusate caused no additional effect (n=6).

Full restoration of pressure could be achieved by the addition of the soluble guanylate cyclase inhibitor, ODQ (20 μM), to the internal perfusate (Fig 5.5). The perfusion pressure in the presence of these compounds was not significantly different from the preconstriction pressure, before addition of NO donor (P=0.73; paired t-test; n=18).

Treatment of control vessels with Hb and ODQ had no significant effect on perfusion pressure (P>0.05; paired t-test; n=6 for both).

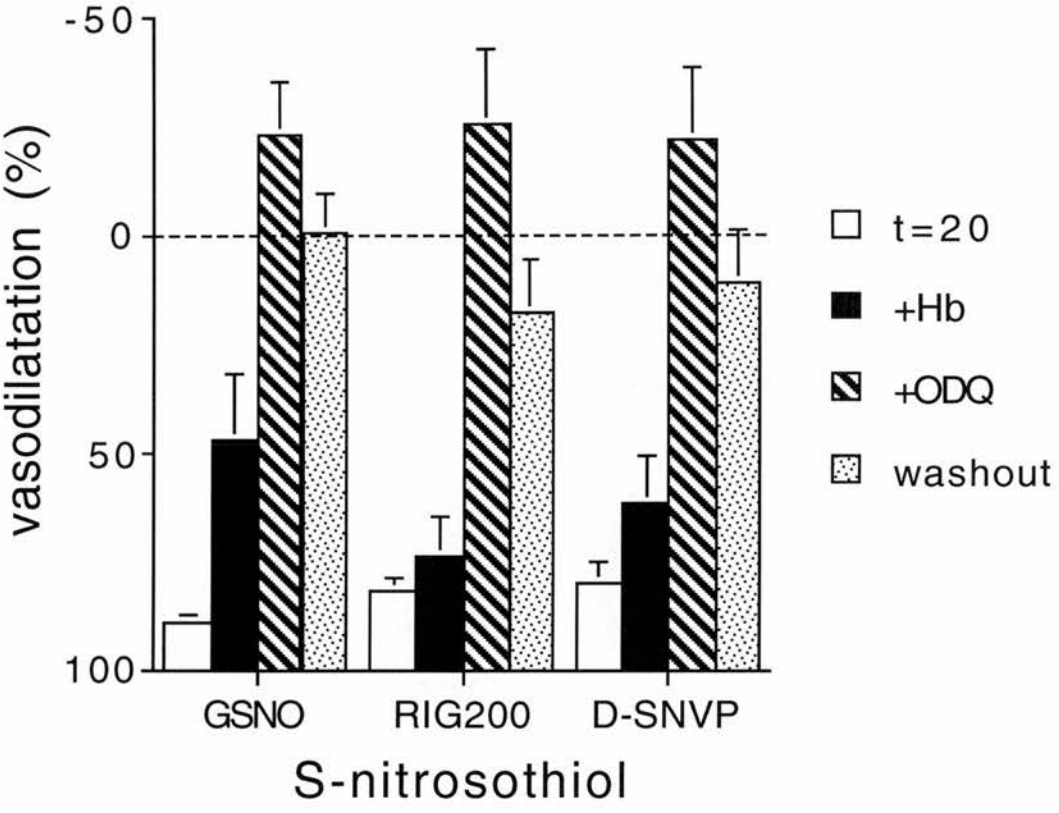


Figure 5.5 The effect of Hb (10 μM), ODQ (20 μM) and S-nitrosothiol washout on the vasodilatation produced by perfusing S-nitrosothiols (10 μM) for 20 h. Points shown are means with vertical lines indicating S.E.M. (n=6 for all). Preconstriction pressure is represented by the dotted line.

5.4 DISCUSSION

The results show that tolerance to GTN develops rapidly in isolated rat femoral arteries within 2-20 h of continuous exposure, resulting in a marked attenuation of the responses to additional bolus concentrations of GTN. The endogenous S-nitrosothiol, GSNO, and novel S-nitrosothiols, RIG200 and D-SNVP, did not induce tolerance within 20 h or exhibit cross-tolerance in vessels made tolerant to GTN.

5.4.1 Model of tolerance

The amplitude of GTN-induced vasodilatation diminished despite continued perfusion of the drug, and was not evident after 20 h. Down-regulation of NO synthase by NO from GTN (Moncada *et al.*, 1991) could be excluded as a possible explanation for the results because the NO synthase inhibitor, L-NAME, was present throughout. Following perfusion with GTN (10^{-5} M), the vasodilator response to boluses of high concentrations of GTN (10^{-4}, 10^{-3} M) were attenuated, confirming nitrate tolerance in these vessels. Perfused isolated femoral arteries are, therefore, an effective *in vitro* model for the investigation of nitrate tolerance and cross-tolerance to NO donor drugs. In addition, tolerance to GTN can be induced rapidly, facilitating studies to investigate the prevention and reversal of nitrate tolerance.

5.4.2 Tolerance profile of S-nitrosothiols

All the S-nitrosothiols (10 μM) that were investigated cause relaxation of arteries to a similar extent, producing ~90% vasodilatation. Vasodilatation to S-nitrosothiols was maintained throughout the 20 h perfusion period, despite the slow decomposition of the compounds in the perfusate reservoir. Our results demonstrate that, despite

structural modifications, RIG200 and D-SNVP retain the characteristics of existing S-nitrosothiols in that they do not engender tolerance (Kowaluk *et al.*, 1987; Kowaluk & Fung, 1990; Matsumoto *et al.*, 1995; Hanspal *et al.*, 2002). Following 20 h S-nitrosothiol perfusion, pressure was rapidly restored by washing out the S-nitrosothiol, confirming the reversibility of the effect and indicating that vessels were still viable. RIG200 and D-SNVP have previously been demonstrated to induce vasodilatation which persists after washout in endothelium-denuded vessels (Megson *et al.*, 1997; Megson *et al.*, 1999). Therefore, the rapid restoration of pressure following S-nitrosothiol washout suggests that the endothelium was functionally intact after 20 h, consistent with the vasoconstrictor effect of L-NAME at the beginning of the experiment.

GSNO-induced vasodilatation was partially inhibited by perfusion with Hb, adding weight to the hypothesis that GSNO releases NO at a site that is susceptible to extracellular NO scavengers (Chapter 3). It also reflects the sensitivity of GSNO to catalyzed decomposition by metal ions in Krebs solution (Dicks *et al.*, 1996; Gordge *et al.*, 1996), or by elements of the vascular cell surface (Kowaluk & Fung, 1990; Al-Sa'doni *et al.*, 1997). The lesser effects of Hb on the vasodilatation to RIG200 and D-SNVP, concurs with the greater stability of these compounds in Krebs solution (Megson *et al.*, 1997; Megson *et al.*, 1999). S-Nitrosothiol-induced vasodilatation was completely reversed by addition of the soluble guanylate cyclase inhibitor, ODQ. As in Chapter 3, this finding suggests that S-nitrosothiols produce a vasodilatation that is predominantly mediated through the NO:sGC pathway.

5.4.3 Consequences for current theories of nitrate tolerance

Our finding that S-nitrosothiols do not induce tolerance implies that the underlying cause of nitrate tolerance *in vitro* is upstream of NO release. Desensitization of the

target enzyme, sGC (Axelsson & Andersson, 1983; Waldman *et al.*, 1986; Mulsch *et al.*, 2001), or upregulation of cGMP-phosphodiesterase activity (Axelsson & Andersson, 1983; Kim *et al.*, 2001) have been suggested as potential mechanisms of tolerance induction. However, our results show that S-nitrosothiols remain fully active in GTN-tolerant vessels. Additionally, the vasodilatation to S-nitrosothiols is predominantly mediated by sGC, alteration of sGC or PDE activity is, therefore, unlikely to be the underlying cause of nitrate tolerance. Tseng *et al* proposed that S-nitrosothiols may be able to activate sGC, through a haem-independent, ODQ-insensitive mechanism that might involve the SH groups of sGC (Tseng *et al.*, 2000). Subsequently, tolerance-induction, and ODQ inhibition (Schrammel *et al.*, 1996), may be mediated by the haem-group of sGC and S-nitrosothiol can bypass this step by transnitrosation of the SH-groups of sGC (Ignarro *et al.*, 1981; Tseng *et al.*, 2000). A number of findings contradict this proposal. Firstly, haem-deficient sGC show little responsiveness to NO (Gerzer *et al.*, 1981; Hobbs, 1997) and redox modulation of the SH groups of sGC has a limited ability to generate cGMP (Braughler, 1983; Kamasaki *et al.*, 1986). Additionally, it is unclear why some compounds that contain a NO^+ moiety are unable to stimulate purified sGC (Dierks & Burstyn, 1996) or, in the case of SNP, produce a vasodilatation that is resistant to ODQ (Brunner *et al.*, 1996; Garcia-Pascual *et al.*, 1999; Homer *et al.*, 1999; Wanstall *et al.*, 2001). Additionally, many studies have shown that NO and NO donors can cause an ODQ-resistant vasodilatation when cGMP levels are abolished (Brunner *et al.*, 1996; Olson *et al.*, 1997; Weisbrod *et al.*, 1998; Homer & Wanstall, 2000). Therefore, it is unlikely that S-nitrosothiols are able to generate sufficient cGMP by a mechanism that is not dependent on the haem-sGC moiety to account for their resistance to tolerance. Overall, sGC-desensitization and PDE upregulation are highly unlikely to be the underlying cause of tolerance.

At present, there is much interest in the role of oxidative stress in tolerance (Munzel *et al.*, 1995; Fink *et al.*, 2000; Kaesemeyer *et al.*, 2000; Munzel *et al.*, 2000;

Loscalzo, 2001; Mulsch *et al.*, 2001; Munzel, 2001; Bassenge *et al.*, 2001; Parker & Gori, 2001; Paulus, 2002). The current results dispute the involvement of superoxide generation in tolerance, because S-nitrosothiols retain their full activity in GTN-tolerant vessels. It could be argued that the S-nitrosothiols used in this study may release NO at a site that is inaccessible to scavenging by superoxide. However, elevated superoxide levels would at least be able to inactivate extracellular NO from GSNO in tolerant vessels. Indeed, the results from Chapter 3 showed that S-nitrosothiol-induced vasodilatation was more susceptible to superoxide generation than GTN, and subsequently if the oxidative stress hypothesis held true, then S-nitrosothiols should be more susceptible to nitrate tolerance than GTN. Additionally, the results from Chapter 4 suggest that if oxidative stress was significantly induced in nitrate tolerance, there would be a non-specific inhibitory effect on vasodilators. The results of other *in vitro* studies also dispute the oxidative stress hypothesis (Laight *et al.*, 1997; Mihm *et al.*, 1999). Munzel *et al* have argue that tolerance-induced superoxide production is more prominent *in vivo* (Munzel *et al.*, 1999), where neurohormonal mechanisms including the renin-angiotensin and endothelin systems may exacerbate oxidative stress (Munzel & Bassenge, 1996). However, the findings of a number of other *in vivo* studies contradict this suggestion (Milone *et al.*, 1999; Ratz *et al.*, 2000; Csont *et al.*, 2001; Minamiyama *et al.*, 2001; Wang *et al.*, 2002). Confusingly, one study has shown that superoxide levels were raised by prolonged GTN treatment *in vitro* (Fink *et al.*, 2000). Although, superoxide levels were not measured in the present study, the contradictory literature suggests that, although oxidative stress may be *associated* with nitrate tolerance, it is not the *underlying cause* of the condition (Mihm *et al.*, 1999; Sage *et al.*, 2000).

In conclusion, the results suggest that events prior to NO release or S-nitrosothiol formation (Ignarro *et al.*, 1981) limit the effectiveness of GTN in tolerance. S-nitrosothiols decompose in physiological solutions at varying rates to generate NO (Williams, 1985) and are able to undergo transnitrosation reactions.

Therefore, S-nitrosothiols are unlikely to be dependent on the same co-factors needed to release of NO from GTN. This property could be the underlying reason why S-nitrosothiols do not induce tolerance, supporting the hypothesis that desensitization or depletion of the nitrate biotransformation system is the underlying cause of tolerance (Brien *et al.*, 1986; Bennett *et al.*, 1988; Bennett *et al.*, 1989; Slack *et al.*, 1989; Feelisch & Kelm, 1991; Sage *et al.*, 2000).

5.4.4 Summary

The two novel analogues of SNAP, RIG200 and D-SNVP, do not induce tolerance with 20 h of continuous perfusion, in an *in vitro* model of tolerance. In addition, they retain full vasodilator potency in vessels made tolerant to GTN, despite its continued presence. Our results lend weight to the argument that RIG200 and D-SNVP may be viable clinical alternatives to organic nitrates and existing S-nitrosothiols, because, added to their previously described increased stability and selectivity for areas of endothelial damage, they do not appear to engender tolerance. These features suggest that RIG200 and D-SNVP could have potential benefits in the treatment of a number of cardiovascular diseases including angina, atherosclerosis, cardiac ischaemia and heart failure where long term vasodilator therapy is required.

Chapter 6

Acetylation of the glucosamine group of RIG200 is required for a marked sustained vasodilatation in endothelium-denuded arteries

6. ACETYLATION OF THE GLUCOSAMINE GROUP OF RIG200 IS REQUIRED FOR SUSTAINED VASODILATATION IN ENDOTHELIUM-DENUDED ARTERIES

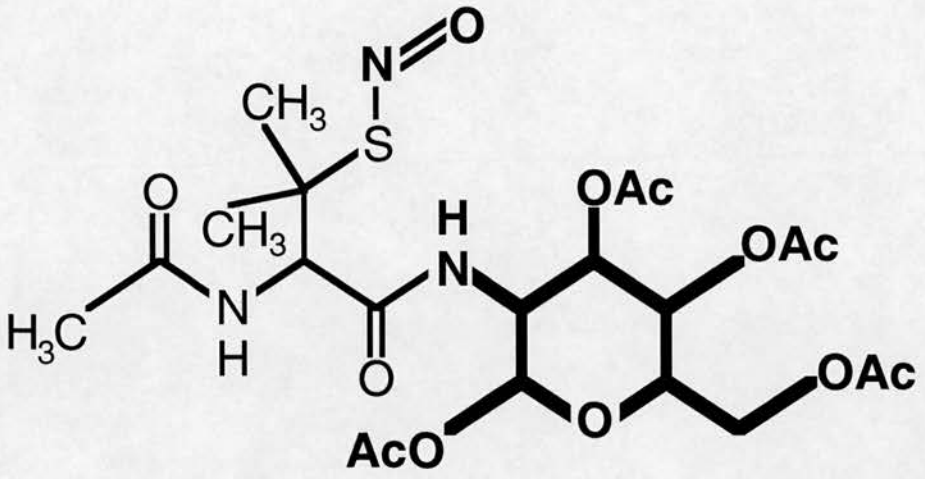
6.1 INTRODUCTION

Recently, Megson *et al* described several novel analogues of the S-nitrosothiol S-nitroso-*N*-acetylpenicillamine (SNAP), including N-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-β-D-glucopyranose (RIG200; SNAP with a acetylated glucosamine group; Megson *et al.*, 1997) and S-nitroso-*N*-valeryl-penicillamine (SNVP; SNAP with a five carbon side-chain; Megson *et al.*, 1999). These structural modifications increased stability by inhibiting metal ion-mediated catalysis through steric hindrance. Additionally, bolus administration of RIG200 to the perfusate of endothelium-denuded isolated arteries caused a vasodilatation that was sustained for a number of hours. A sustained effect was not seen in parallel experiments with RIG200 in endothelium-intact vessels, or with SNAP, irrespective of the integrity of the endothelium. The sustained vasodilatation was reversed by the NO scavenger, Hb, but not by the NOS inhibitor, L-NAME, indicating that the vasodilatation was mediated by NO that was not derived from NOS (Megson *et al.*, 1997; Megson *et al.*, 1999).

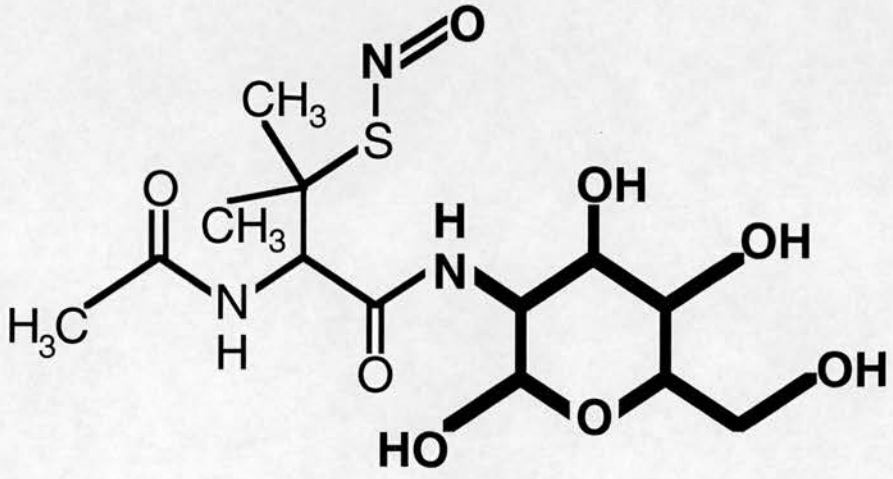
N-substituted analogues of SNAP with increasing lengths of alkyl side chains were found to have increasing lipophilicity (Megson *et al.*, 1999). In similar experiments to those with RIG200, strong correlation was observed between

lipophilicity and sustained vasodilatation (see Fig 1.21). The authors hypothesised that the endothelium acts as a barrier to these compounds, resulting in transient responses to bolus injections that recover rapidly once the bolus is washed out of the lumen of the vessel. Endothelial denudation removes the barrier, allowing lipophilic S-nitrosothiols to gain access to lipid-rich sub-endothelial structures, where they are retained and slowly decompose to NO, producing a sustained vasodilatation (see Fig 1.22).

Although, it has been established that lipophilicity affects the ability of N-substituted analogues of SNAP to cause sustained vasodilatation, it has not been determined whether the same property affects the vasodilator activity of glucosamine-containing analogues of SNAP. RIG200 contains a acetylated glucosamine group. Removal of the acetyl groups generates a highly water-soluble compound, N-(S-nitroso-N-acetylpenicillamine)-2-amino-2-deoxy-α,β-D-glucopyranose (glyco-SNAP; Ramirez *et al.*, 1996; Fig 6.1), which retains the glucosamine group. Here, isolated rat femoral arteries were used to test the hypothesis that, unlike RIG200, bolus administration of glyco-SNAP fails to cause sustained vasodilatation in endothelium-denuded arteries.



_N_-(_S_-nitroso-_N_-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-_O_-acetyl-β-D-glucopyrnaose (RIG200)



_N_-(_S_-nitroso-_N_-acetylpenicillamine)-2-amino-2-deoxy-α,β-D-glucopyranose (glyco-SNAP)

Figure 6.1 Chemical structure for RIG200 and glyco-SNAP. Area highlighted in bold represents the glucosamine group that is acetylated in RIG200, but not in glyco-SNAP.

6.2 METHODS

6.2.1 Preparation

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (250-350 g; n=19) in a perfusion system. The vessels were perfused (0.6 ml min^{-1}) and superfused (1 ml min^{-1}) with fresh oxygenated (95% O_2, 5% CO_2) Krebs buffer solution. Vessel tone was measured by monitoring perfusion pressure with a differential pressure transducer. All experiments were carried out in a darkened laboratory (see Section 2.3.2).

6.2.2 Endothelial denudation

Several vessels were denuded of their endothelium prior to cannulation and mounting in the perfusion system (Megson *et al.*, 1997). Following dissection, a stainless steel wire was passed through the lumen and then removed. Loss of the endothelium was confirmed by showing that sub-maximally constricted vessels (2 μM) were no longer responsive to Hb (10 μM) or L-NAME (20 μM).

6.2.3 Experimental protocol

6.2.3.1 Preconstriction

Vessels were preconstricted with phenylephrine (PE; 2 μM) prior to treatment with a supramaximal concentration of the NO synthase inhibitor L-NAME (20 μM). Following 40 min L-NAME treatment, additional PE (2-8 μM) was added to obtain a final preconstriction pressure of 120-150 mmHg (Fig 6.2).

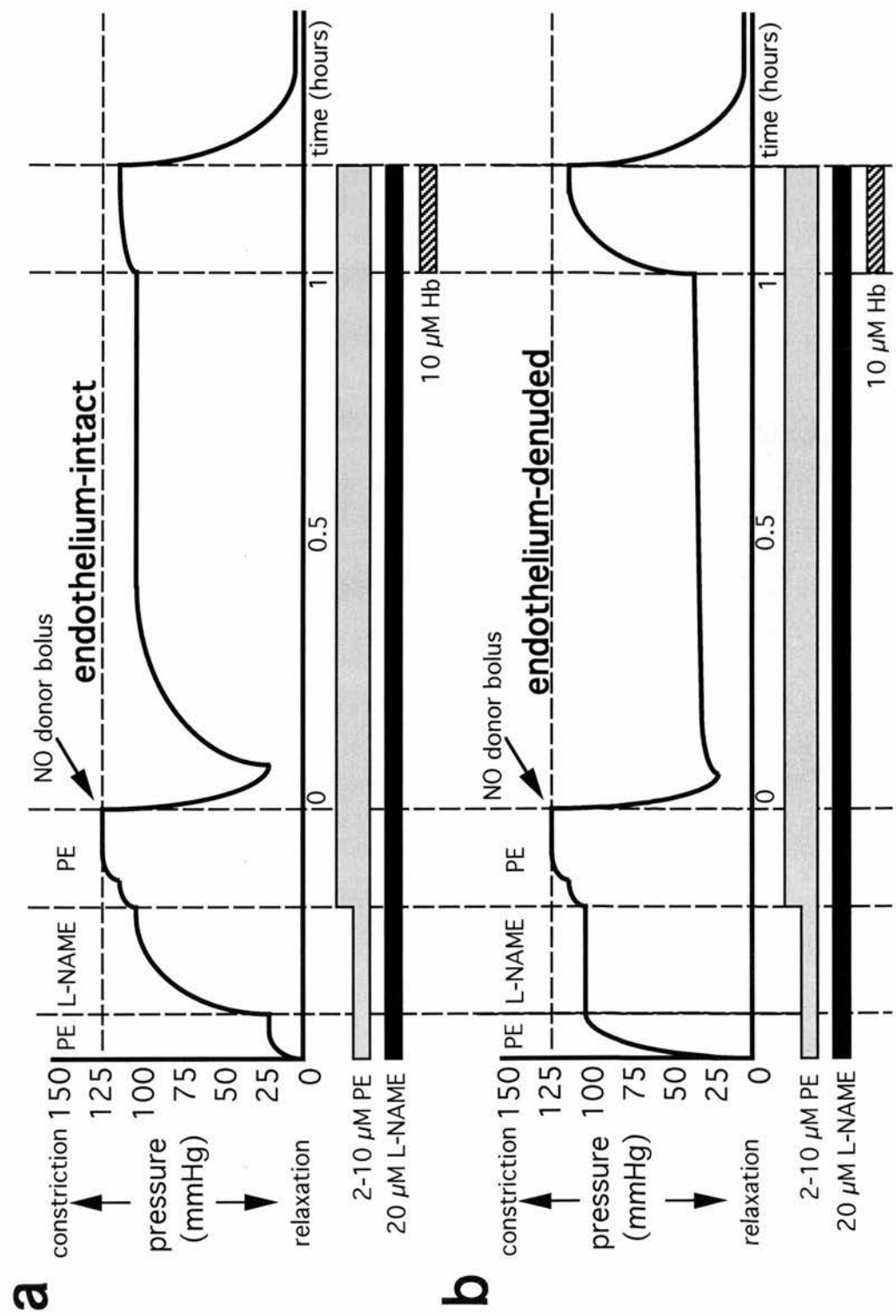


Figure 6.2 Schematic diagram for the protocol used in this study. (a) Endothelium-intact arteries, (b) endothelium-denuded arteries. Note that NO donors are administered as a single bolus and are not perfused over long periods of time.

6.2.3.2 Administration of NO donor

Following preconstriction, a bolus (10 μl; 10^{-3} M) of either RIG200 or glyco-SNAP was administered into the perfusate. Previous experiments (Megson *et al.*, 1997) have shown that drug boluses pass through the vessel lumen in <1 s. Perfusion pressure was monitored for 1 h after administration. At this time, Hb (10 μM) was added to the internal perfusate to reverse NO-mediated vasodilatation (Fig 6.2).

6.2.4 Analysis of Results

Vasodilator response amplitude was expressed as a % of (PE+L-NAME)-induced pressure existing before drug delivery (% pressure change; positive values represent vasodilatation, where 100% represents complete abolition of agonist-induced tone). Mean values are given ± S.E.M..

P-values in the text were obtained by two-tailed paired and unpaired Student's *t*-tests.

6.3 RESULTS

6.3.1 Preconstriction

In endothelium-intact arteries, 2 μM PE caused a vasoconstriction of 23.1±2.1 mmHg (n=17). L-NAME (20 μM) caused further vasoconstriction to generate a pressure of 77±9 mmHg (n=17). In endothelium-denuded arteries, 2 μM PE produced a vasoconstriction of 90±10 mmHg (n=16) that was unaffected by L-NAME.

Additional PE (2-8 μM) was added to obtain a final pressure of 120-150 mmHg. The final pressure in endothelium-intact arteries (136±6 mmHg) was not significantly different from that of endothelium-denuded arteries (121±10 mmHg; P=0.19; unpaired t-test; n=16-17).

6.3.2 Vasodilatation to NO donors

Bolus injections of RIG200 (10 μl; 10^{-3} M) produced a maximum vasodilator response of 68±3% (n=6) in endothelium-intact arteries and 77±3% (n=6) in endothelium-denuded arteries. Perfusion pressure in endothelium-intact arteries recovered rapidly to ~35 % vasodilatation within 5 min, whereas in endothelium-denuded arteries a vasodilatation of ~65 % remained after 5 min and persisted throughout the 1 h perfusion period. Vasodilatation remaining at 1 h was significantly greater in endothelium-denuded arteries (51±5%) than in endothelium-intact arteries (21±7%; P=0.01; unpaired t-test; n=6; Fig 6.3).

Bolus injections of glyco-SNAP produced similar responses in endothelium-intact and -denuded arteries. Peak vasodilatation was 68±4% in both intact and denuded arteries, (n=6 and 8, respectively). Vasodilatation remaining at 1 h was not significantly different in endothelium-intact arteries (20±3%) compared to endothelium-denuded arteries (25±7%; P=0.55; unpaired t-test; n=6-8; Fig 6.3).

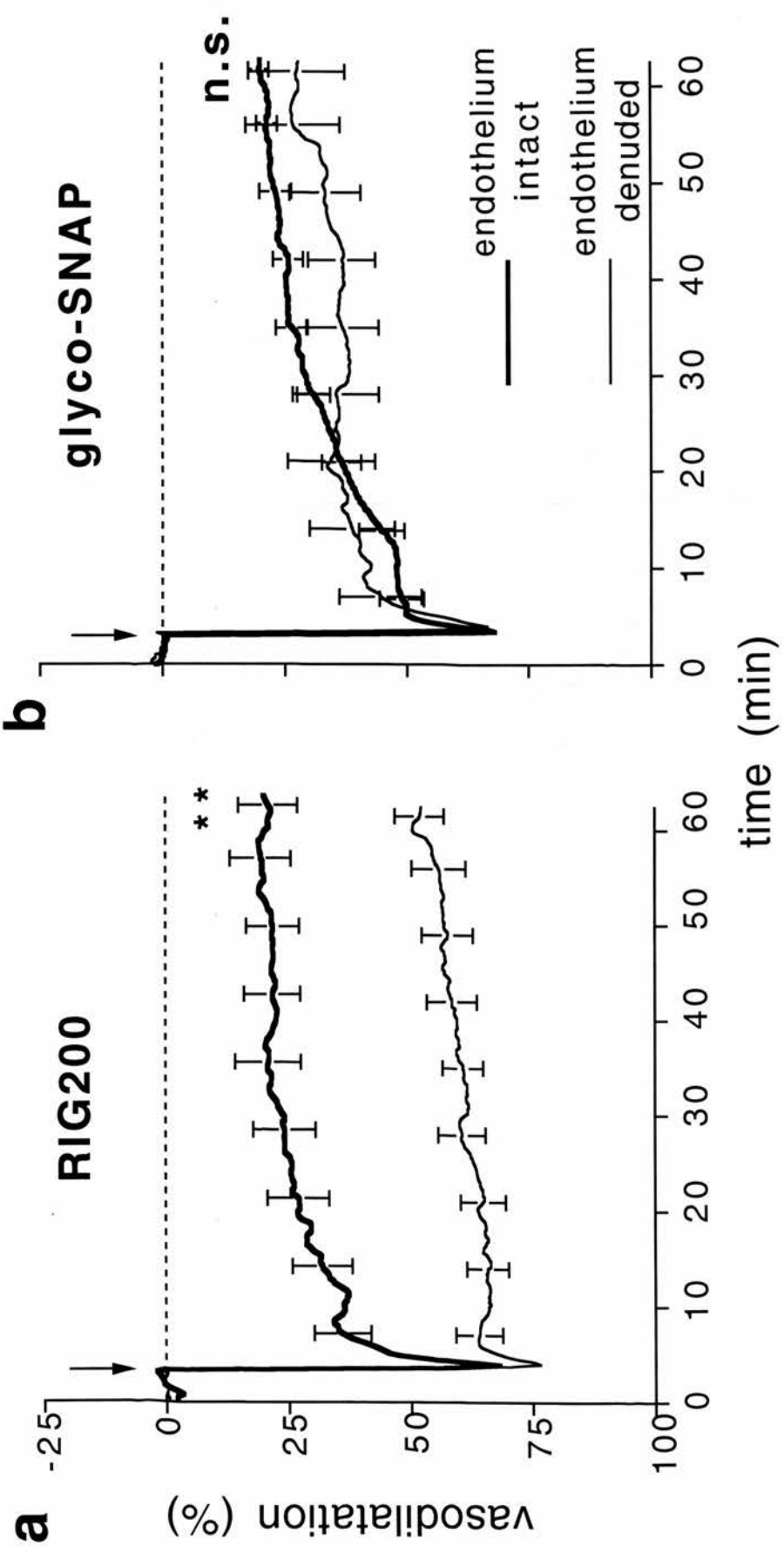


Figure 6.3 Vasodilatation caused by (a) RIG200 and (b) glyco-SNAP in endothelium-intact (bold line) and endothelium-denuded (thin line) arteries. Lines represent a mean of 6-8 experiments, with S.E.M. represented by error bars, shown every 6 minutes. Arrows show time of NO donor bolus administration.

6.3.3 Hb-induced reversal of vasodilator responses

Hb (10 μM) had no significant effect on the vasodilator responses to RIG200 that remained after 1 h in endothelium-intact arteries (P=0.37; paired t-test; n=5). However, Hb partially reversed the sustained vasodilatation to RIG200 in endothelium-denuded arteries (P=0.008; paired t-test; n=5; Fig 6.4).

Hb (10 μM) completely reversed the remaining vasodilatation to glyco-SNAP at t=1 h, although, this reversal was only significant in endothelium-denuded (P<0.001; paired t-test; n=8), and not in endothelium-intact (P=0.14; paired t-test; n=6) arteries (Fig 6.4).

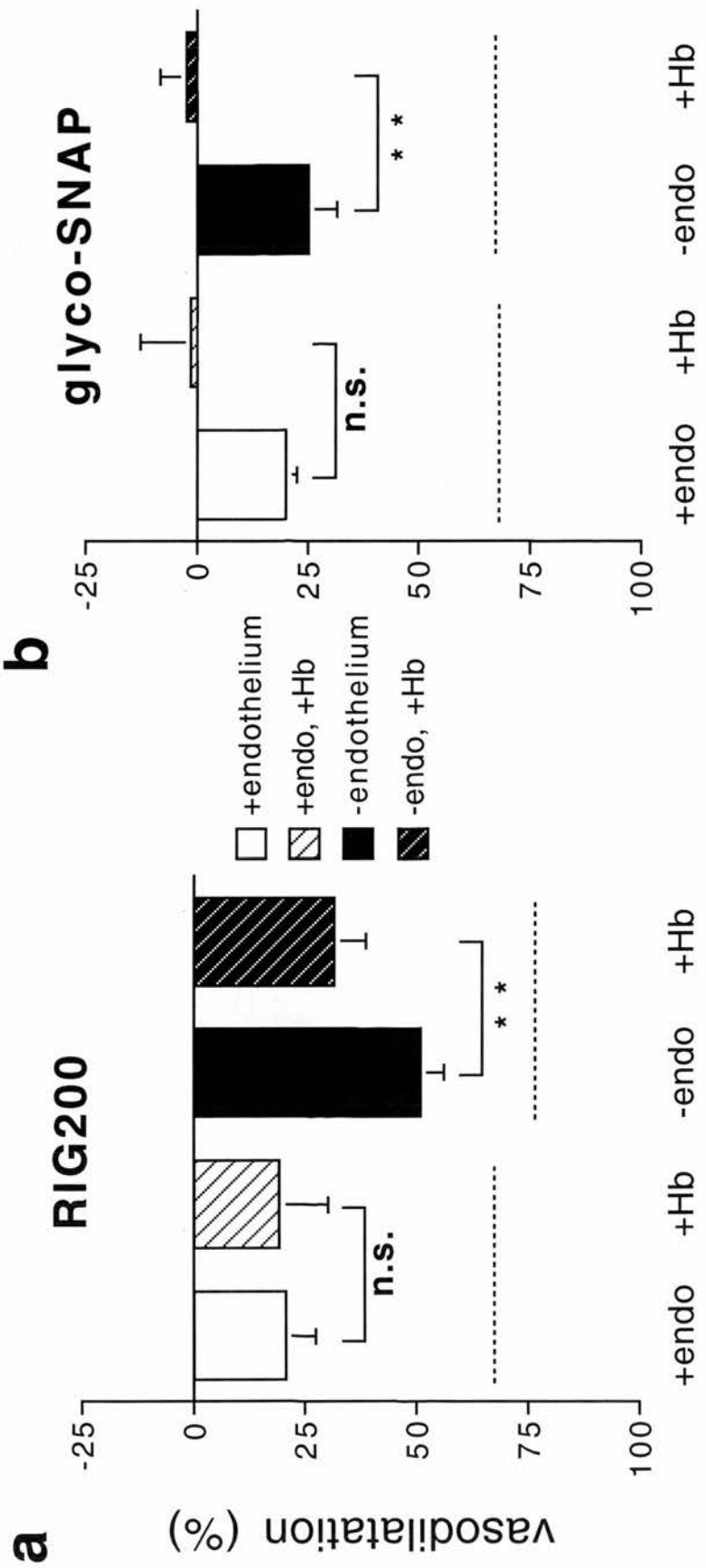


Figure 6.4 Reversal of the vasodilatation at t=1 h by Hb (10 μM). (a) RIG200, (b) glyco-SNAP in endothelium-intact (open columns) and endothelium-denuded (filled columns) arteries. Dotted line represents to maximum vasodilatation caused by boluses of NO donors (t=0 h).

6.4 DISCUSSION

RIG200 causes a marked NO-mediated sustained vasodilatation (~50%) in endothelium-denuded blood vessels, but not endothelium-intact, isolated rat femoral arteries. A small (~20%) non-specific element of this effect is seen in endothelium-intact vessels and, therefore, this component is not selective for endothelial denudation. Glyco-SNAP, an analogue of RIG200 containing a non-acetylated glucosamine group, causes only a modest sustained vasodilatation (~20%) that is not dependent on endothelial denudation and is entirely NO mediated.

6.4.1 Ability to induce sustained vasodilatation

Bolus administration of RIG200 to the lumen of isolated perfused endothelium-intact femoral arteries, produces a transient vasodilatation. As shown previously (Megson *et al.*, 1997), an identical bolus of RIG200 administered to endothelium-denuded arteries, produces a prolonged vasodilatation, despite the apparent washout of the bolus in <1 sec. In the current study, perfusion pressure only returned to ~50% of the preconstriction level 1 h after the bolus injection, equating to ~66% of maximum vasodilatation observed immediately after bolus injection. The sustained vasodilator actions of RIG200 in denuded blood vessels have also been observed *in vitro* in human arteries and veins used for bypass grafting in coronary artery disease (Sogo *et al.*, 2000) and *in vivo* in human hand vein (Sogo *et al.*, 2000).

Other analogues of SNAP with N-substituted carbon side-chains have also been shown to produce a sustained vasodilatation in isolated perfused femoral arteries (Megson *et al.*, 1999). The degree of sustained vasodilatation was shown to strongly correlate with the lipophilicity of the compound (Megson *et al.*, 1999). Glyco-SNAP is composed of SNAP linked to glucosamine (Ramirez *et al.*, 1996). This compound is

identical to RIG200, except that the acetyl groups have been removed from the glucosamine moiety. Acetylation of compounds increases their lipophilicity and, consequently, glyco-SNAP represents a highly water-soluble analogue of RIG200. As expected, glyco-SNAP was unable to cause a substantial sustained vasodilatation in endothelium-denuded arteries. These results support the hypothesis that the lipophilicity of S-nitrosothiols is a major factor in facilitating sustained vasodilatation in blood vessels with a damaged endothelium.

6.4.2 Mechanism of sustained vasodilatation

Previously, it had been shown that the sustained vasodilatation produced by RIG200 and SNVP was reversible with the NO scavenger, Hb, but not by the NOS inhibitor, L-NAME. Subsequently, it has been hypothesised that the endothelium acts as a barrier to S-nitrosothiols, allowing only transient vasodilatation due to the release of NO as the drug bolus passes through the blood vessel. However, results from experiments with endothelium-denuded arteries are consistent with the theory that lipophilic S-nitrosothiols are retained in lipid-rich regions, where they slowly decompose to NO, causing sustained vasodilatation that is susceptible to scavenging by Hb.

In the current study, the sustained vasodilatation induced by RIG200 was only partially reversed by Hb. At present, it is not clear why Hb was unable to completely reverse vasodilatation. The small (~20%) Hb-resistant effect may not be due to the glucosamine group of RIG200, because responses to SNAP, a compound that does not contain this group, have previously been shown to cause similar vasodilatation in endothelium-intact arteries 1 h after bolus administration (Megson *et al.*, 1997). Given that this effect is only seen at high concentrations, it is possible that the remaining vasodilatation to RIG200 is due to an sGC-independent mechanism. However, the

results of Chapter 3 demonstrate that sGC-independent vasodilatations caused by S-nitrosothiols are practically abolished by extracellular scavengers such as Hb.

Assuming that the Hb-resistant vasodilator effect of RIG200 is not due to an unidentified non-specific action of the structure of the compound, the most likely explanation is that the high lipophilicity of RIG200 facilitates its retention in extracellular areas that Hb cannot access. RIG200 may even enter cells, as has been suggested for lipophilic analogues of SNO-Cys (Clancy *et al.*, 2001), perhaps explaining why RIG200 also causes a small sustained Hb-resistant vasodilatation in endothelium-intact vessels. Membrane bound glucose transporters may also be involved in the transport of sugar-linked S-nitrosothiols into cells (Cantuaria *et al.*, 2000).

A small residual vasodilator effect of glyco-SNAP was also seen at 1 h. Although this vasodilatation was of a similar size to that for RIG200 (~25%), it is unlikely to be caused by its retention in the *same* compartments as RIG200, because glyco-SNAP is a highly water-soluble compound (Ramirez *et al.*, 1996). However, the finding that Hb can reverse the vasodilatation supports a role for NO in this effect. At present, the most likely explanation is that this vasodilatation is caused by small concentrations of glyco-SNAP that become associated with hydrophilic regions of the endothelial or sub-endothelial surface. Generation of NO at this site is susceptible to Hb, as glyco-SNAP is not sufficiently lipophilic to penetrate deeper into the tissue, where Hb cannot penetrate (Foley *et al.*, 1993). This hypothesis could be tested in similar experiments to the one described here, using radiolabelled S-nitrosothiols.

6.4.3 Summary

This study demonstrates high lipophilicity is required for S-nitrosothiols, such as RIG200, to cause a marked sustained vasodilatation in endothelium-denuded vessels. These results suggest that lipophilic S-nitrosothiols may be able to produce sustained

NO-mediated effects selectively at areas of endothelial-damage, from a single bolus administration. This is a particularly attractive feature in the treatment of cardiovascular conditions where the endothelium may be damaged, such as atherosclerosis, or following interventional techniques, such as bypass grafting and balloon angioplasty.

Chapter 7

A novel S-nitrosothiol with prolonged actions at sites of vascular injury selectively inhibits platelet adhesion in rabbit carotid arteries following balloon angioplasty

7. A NOVEL S-NITROSOTHIOL WITH PROLONGED ACTIONS AT SITES OF VASCULAR INJURY SELECTIVELY INHIBITS PLATELET ADHESION IN RABBIT CAROTID ARTERIES FOLLOWING BALLOON ANGIOPLASTY

7.1 INTRODUCTION

Percutaneous transluminal coronary angioplasty is a common clinical intervention used to improve blood flow through stenosed coronary arteries, although its long-term benefit is limited by restenosis. The underlying causes of restenosis have not yet been fully characterised, but proliferation and vascular remodeling are evident within a few days of injury (Wei Liu *et al.*, 1989; Lincoff *et al.*, 1994). The procedure inevitably damages the vascular endothelium, leading to activation and adhesion of platelets within an hour of angioplasty (Clowes & Karnovsky, 1977; Groves *et al.*, 1979; Wei Liu *et al.*, 1989). Platelet adhesion to the exposed sub-endothelium and release of their cytoplasmic granules are a key event in the initiation of restenosis (Friedman *et al.*, 1977; Goldberg & Stemerman, 1980; Fingerle *et al.*, 1989).

Current anti-thrombotic therapies reduce the incidence of thrombosis and restenosis. However, their use is limited by undesirable systemic effects and, despite current advances in drug delivery catheters and deployment of stents, endothelial

damage and neointimal hyperplasia remain major problems leading to a 15-30% incidence of restenosis (Topol & Serruys, 1998; Bult, 2000; Swanson *et al.*, 2001).

Loss of the protective effects of endothelium-derived nitric oxide (NO) is critical to increased platelet adhesion. NO has powerful anti-platelet actions and NO donors can reduce platelet activation (Langford *et al.*, 1994) and adhesion (Lam *et al.*, 1988; Groves *et al.*, 1993) following angioplasty. However, existing NO donor drugs are not selective for areas of endothelial damage and dosing is limited by systemic hypotension. In contrast, lipophilic S-nitrosothiol NO donor drugs have a prolonged vasodilator activity that is selective for blood vessels with experimentally denuded endothelium (Megson *et al.*, 1997; Megson *et al.*, 1999).

Using a rabbit model, this study tests the hypothesis that platelet adhesion to the intimal surface of common carotid arteries following angioplasty is inhibited to a greater extent by SNVP than by the conventional nitrate, GTN.

7.2 METHODS

7.2.1 Surgical procedure

Adult male New Zealand white rabbits (2.5-3.5 kg; n=60) were anaesthetised as described in Section 2.4. The left femoral artery was cannulated for the measurement of systemic blood pressure (BP) and heart hate (HR) throughout the procedure and withdrawal of blood. Angioplasty was performed on a 40 mm section of the common carotid artery. Sham operations involved cannulation of the common carotid artery without balloon inflation. The carotid artery was recannulated to administer a 0.2 ml bolus of heparinised saline (Hep-Sal; 25 U/ml), GTN or SNVP (both 200 nmoles) immediately upstream of the angioplastied region (Fig 7.1, 7.2). Both SNVP (Megson *et al.*, 1999) and GTN (Bennett *et al.*, 1989) release 1 molar equivalent of NO. Blood flow over the angioplastied region was restored and animals were killed 35 min after angioplasty. Both common carotid arteries were dissected free and divided into rings for further assessment (Fig 7.1; See Section 2.4).

7.2.2 Preparation and measurement of radiolabelled platelets

Citrated blood was centrifuged with prostacyclin (PGI_2; 300 ng/ml) to obtain pelleted platelets. Platelets were radiolabelled with $^{111}InCl_3$ and re-suspended in PGI_2-free solution (Norman *et al.*, 1997). Approximately 1 ml platelet suspension (50-300 $x10^6$ platelets; radioactivity=50-800 $x10^3$ decays per minute; dpm) was re-injected into the donor rabbit via a marginal ear vein ~10 min before angioplasty. Radioactivity of blood samples (100 µl) and segments of carotid artery (~5 mm) were assessed using a liquid scintillation analyser (see Section 2.6).

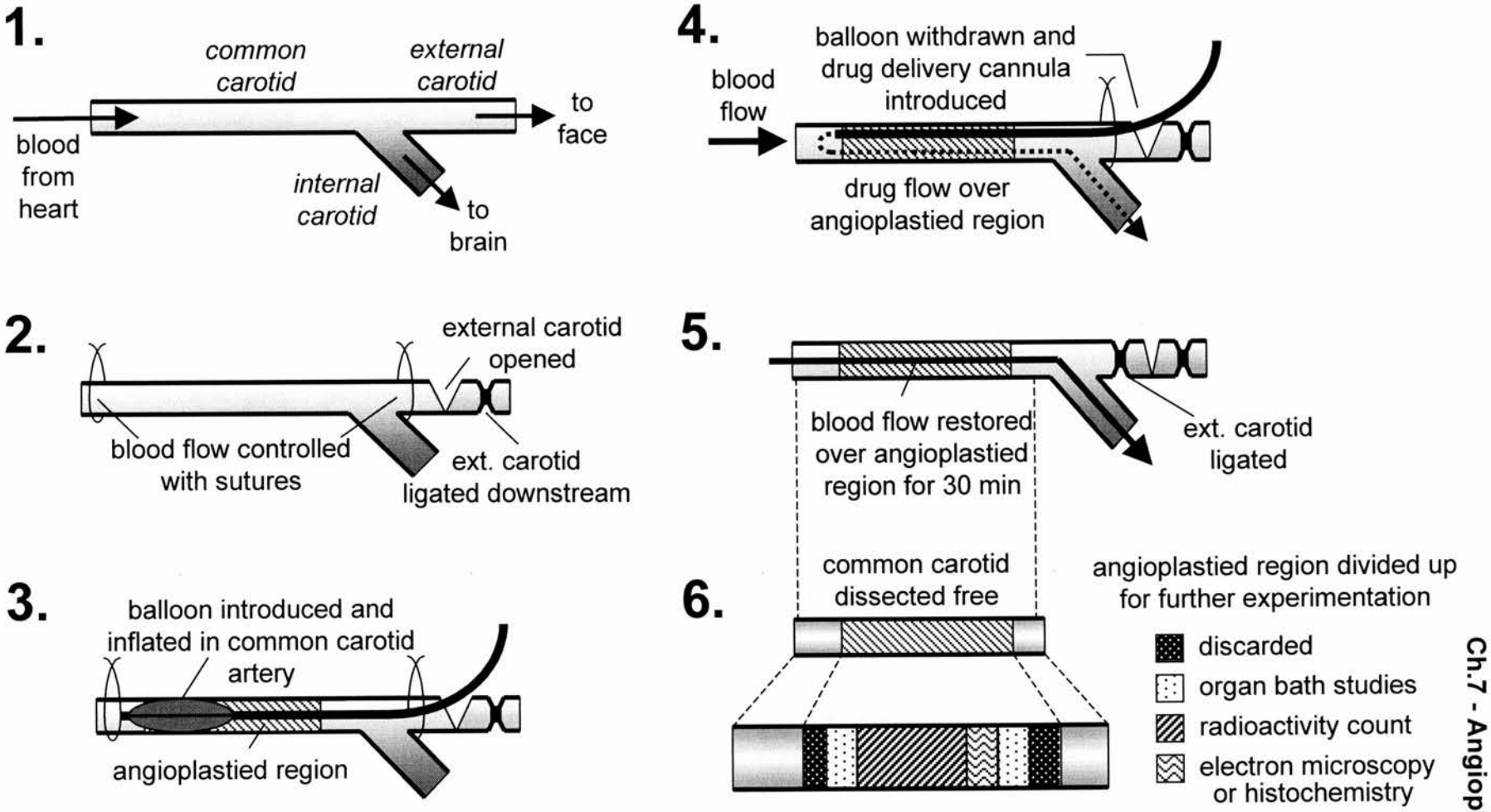


Figure 7.1 Schematic diagram of the treatment and utilisation of the common carotid artery in this study. The hatched area represents the angioplastied region, which is isolated and divided up, as indicated, for further experimentation.

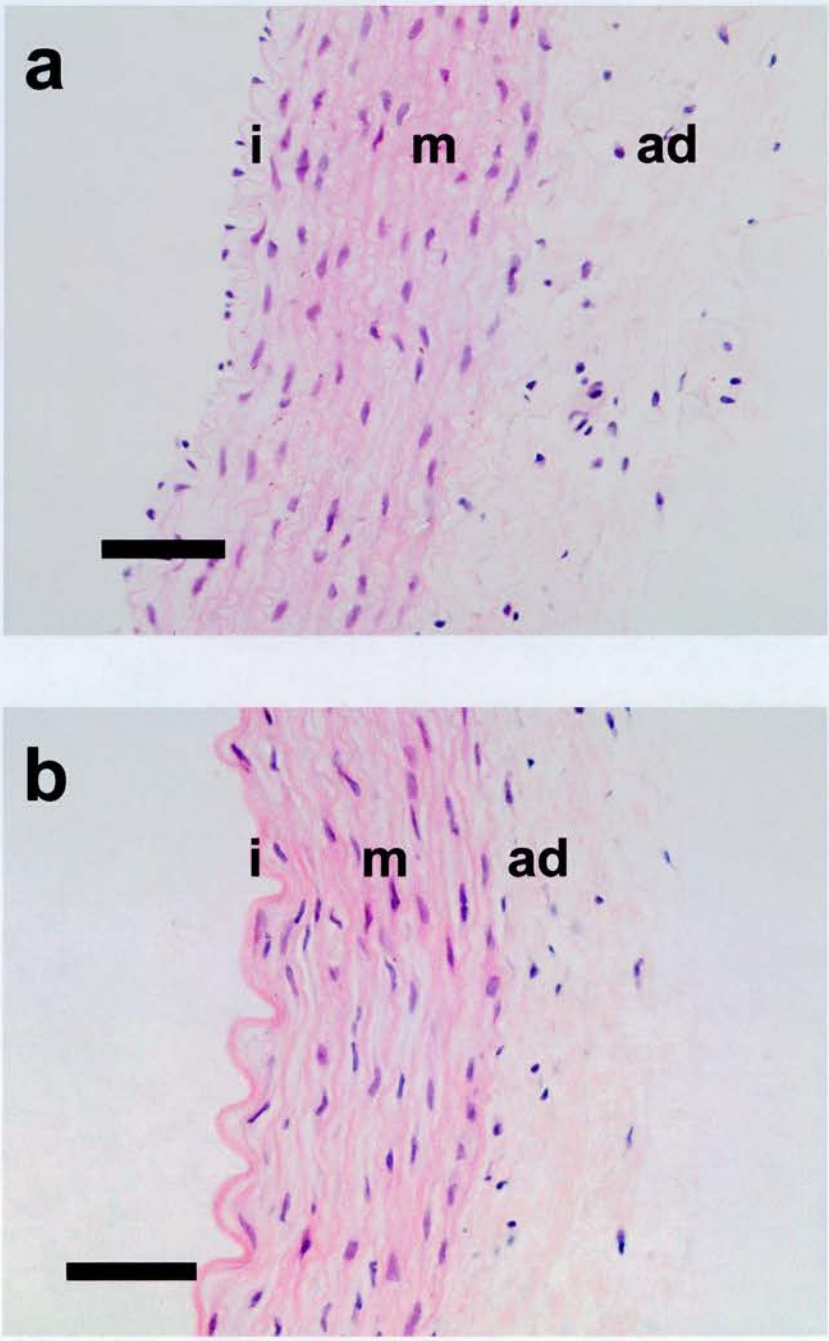


Figure 7.10 Representative images of sections of carotid arteries, with cell nuclei stained with haematoxylin (purple) and general tissue structure stained with eosin (pink). (a) Contralateral (uninjured) artery. (b) Angioplastied artery; note that there are no nucleated cells in the intimal layer (i). Media (m), adventitia (ad). Vessels were isolated and fixed 30 minutes after drug treatment. Black bar represents 50 μm.

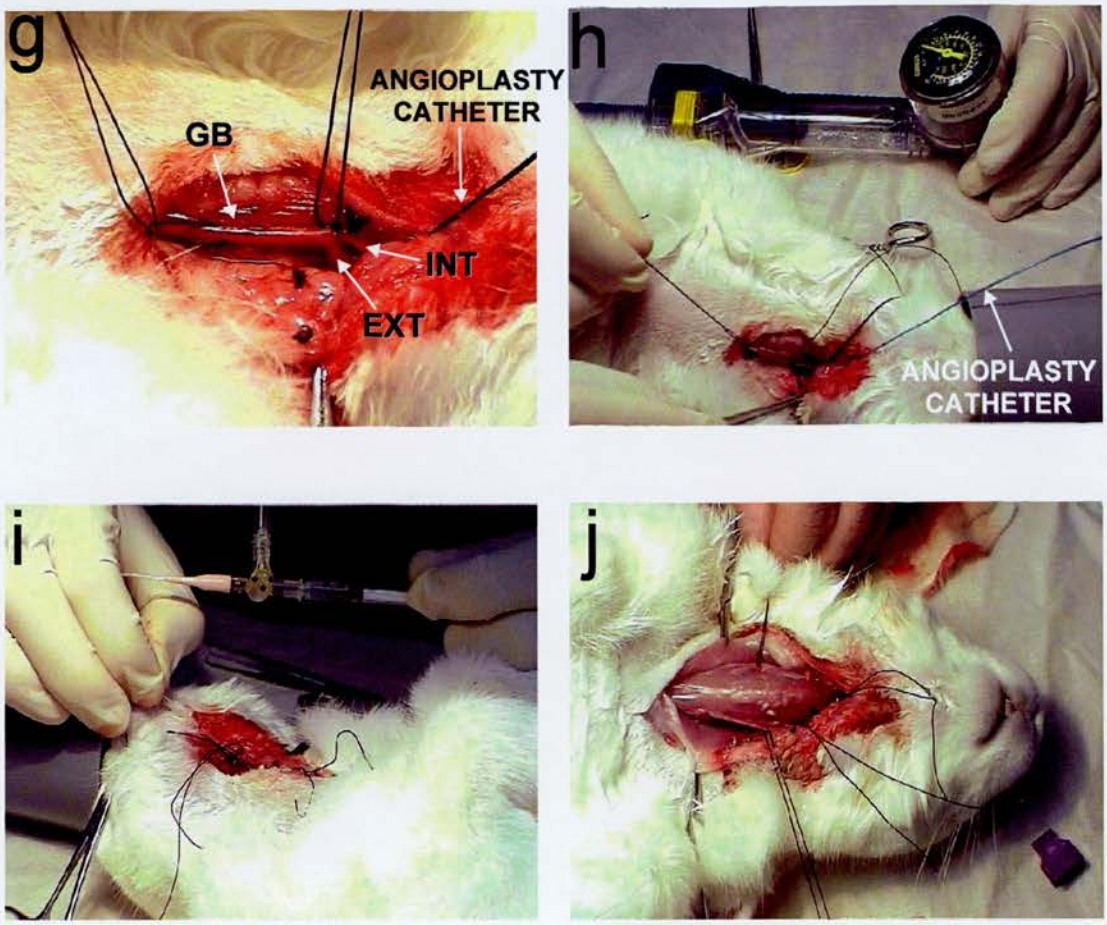


Figure 7.2 Photographic stills of the surgical procedure. (a) Angioplasty catheter. Ruler and one pence piece used as indicators of size. (b) Tips of two angioplasty catheters, with inflated (top) and deflated (bottom) balloon. (c) exposed left femoral artery. The artery (FA; red) has been isolated with suture, in between the femoral nerve (FN; white) and the femoral vein (FV; purple). (d) cannulated femoral artery is attached to a transducer to measure systemic blood pressure. (e) Initial incision to the neck. Anaesthesia is maintained by inhaled halothane through the grey funnel. (f) Exposed left common carotid artery. The bifurcation of the common carotid artery (COM) is clearly seen, with the internal (INT) and external (EXT) carotid arteries also visible. (g) Following injection of radiolabelled platelets via the marginal ear vein, the deflated angioplasty balloon is introduced into the external carotid artery and passed into the common carotid artery. The gold band (GB) at the tip of the balloon can be seen faintly through the common carotid artery. (h) the balloon is passed 1 cm beyond the regions that might have been damaged by sutures controlling blood flow, and inflated twice. (i) The Angioplasty balloon is removed and the external carotid artery is recannulated for drug delivery. Boluses are administered immediately upstream of the angioplastied region. (j) Drug bolus cannula is removed, the external carotid is sutured and blood flow is restored through the common carotid. Thirty min after drug administration, the animal is sacrificed, both left and right common carotid arteries are fully exposed and isolated for further experimentation.

7.2.3 Platelet aggregation

Five ml blood samples were taken from the femoral artery before angioplasty and immediately before the animal was killed. Platelet-rich plasma (PRP; 0.5 ml) was pre-warmed to 37°C for 5 min in a two-channel platelet aggregometer. Platelet aggregation in response to supramaximal concentrations of adenosine 5'-diphosphate (ADP; 8 μM) was measured turbidometrically (Megson *et al.*, 2000; see Section 2.7).

7.2.4 Plasma catecholamines

Plasma catecholamines in samples were measured before and after angioplasty and adminstration of NO donors (Sedowofia *et al.*, 1998; see Section 2.8).

7.2.5 Organ bath studies

Vessel rings (3 mm) were suspended in a 10 ml myograph and bathed in Krebs solution at 37°C. Tension was applied to vessels in stepwise increments to obtain a resting tension of 7 g (Dong *et al.*, 1997) and allowed to equilibrate for 30-40 min. Rings were contracted (x3) to obtain the maximum contraction to high K^+ Krebs (NaCl: 4.7 mM; KCl: 118 mM). Rings were exposed to PE (0.1-10 μM) to investigate the effect of *in vivo* NO donor administration on blood vessel contractility. Following precontraction with EC_{80} PE (~3 μM), endothelial cell function was assessed using ACh (0.01-30 μM). Vessels were precontracted with EC_{50} PE (~1 μM) and the response to the NO scavenger oxyhemoglobin (Hb; 10 μM; to inhibit both endogenous and exogenous NO) and the NO synthase inhibitor L-NAME (200 μM; to inhibit only endogenous NO) was measured (see Section 2.5).

7.2.6 Electron microscopy (EM)

Segments of carotid artery (3 mm) were fixed in 3% glutaraldehyde and osmium tetroxide and examined by scanning and transmission electron microscopy (see Section 2.9).

7.2.7 Histochemistry and immunohistochemistry

Segements of carotid artery were fixed in formalin (10%) and embedded in paraffin wax prior to sectioning (3 μm). Sections were stained using haematoxylin and eosin or unconjugated Griffonia (Bandeiraea) Simplicifolia Lectin I (GSL; see Section 2.10)

7.2.8 Analysis of results

In organ bath experiments, changes in tone are expressed as a % of maximal response to the third administration of high K^+ Krebs. Changes in BP/HR were expressed as maximum % change in BP/HR existing before drug bolus administration. Results from aggregation studies are expressed as the change in aggregation of platelets withdrawn immediately before sacrifice, compared to those withdrawn before angioplasty. Radiolabelled-platelet adhesion was expressed as an index, standardised by vessel length (cm) and whole blood radioactivity (decays per minute/100 μl blood). Thus, an index of 1.0 indicates that all the radiolabelled platelets in 100 μl of blood adhered to a 1 cm segment of vessel. Mean values are given ± S.E.M.. ANOVAs and paired and unpaired Student's *t*-tests were used where appropriate.

7.3 RESULTS

7.3.1 Effect of angioplasty on vessel function

Compared to contralateral control vessels, angioplastied vessels showed a 29±8% decrease in vasoconstriction to high K^+ Krebs (P=0.02; paired t-test; n=6; Fig 7.3a). In addition, vasoconstrictor responses to PE (0.1-10 μM) were also attenuated (P=0.002; 2-way repeated-measures ANOVA; n=6; Fig 7.3b). Responses to high K^+ Krebs and PE in sham-operated vessels were not different from those in contralateral vessels (P=0.36; paired t-test; n=7 and P=0.68; 2-way repeated-measures ANOVA; n=7, respectively).

Following precontraction with PE (EC_{80};2.7±0.2 μM; n=12), ACh (0.01-30 μM) produced a similar degree of concentration-dependent vasodilatation in un-injured contralateral and sham vessels, with almost complete loss of tone with 30 μM ACh (Fig 7.4a). There was marked attenuation of the maximum vasodilatation to ACh in angioplastied vessels, from 90±2 to 20±10% (P<0.001; 2-way repeated-measures ANOVA; n=6).

In the presence of PE (EC_{50};1.3±0.1 μM; n=11), Hb (10 μM) increased tone from 54±3% to 92±5% (n=7) of the maximal K^+ Krebs-induced contraction in the contralateral vessels (Fig 7.4b). In the same vessels, L-NAME (200 μM) increased vessel tone from 59±1% to 73±2% (n=6; Fig 7.4b). The responses to Hb and L-NAME were similar in sham-operated in comparison to contralateral vessels (P=0.37 and P=0.06, respectively; paired t-test; n=6-7). In angioplastied vessels, Hb and L-NAME had no effect on vessel tone (P=0.82 and P=0.43, respectively; paired t-test; n=3-5).

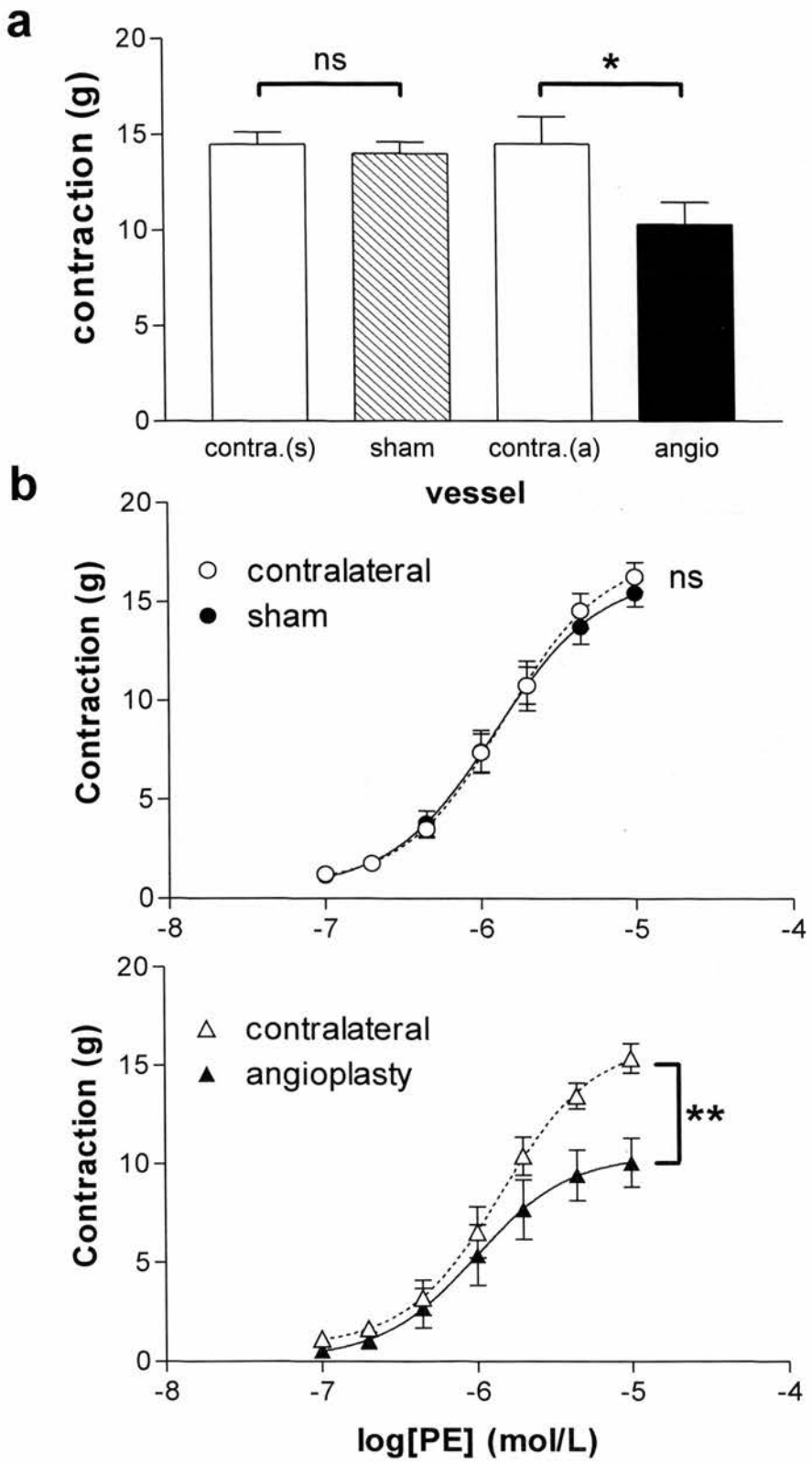


Figure 7.3 Vasoconstrictor responses in carotid rings from sham-operated (hatched column/filled circles), angioplastied (filled column/filled triangles) and their corresponding contralateral (open columns/open symbols) vessels to (a) high K^+ (118 mM) Krebs and (b) PE (0.1-10 μM). Mean±S.E.M. (n=6-7).

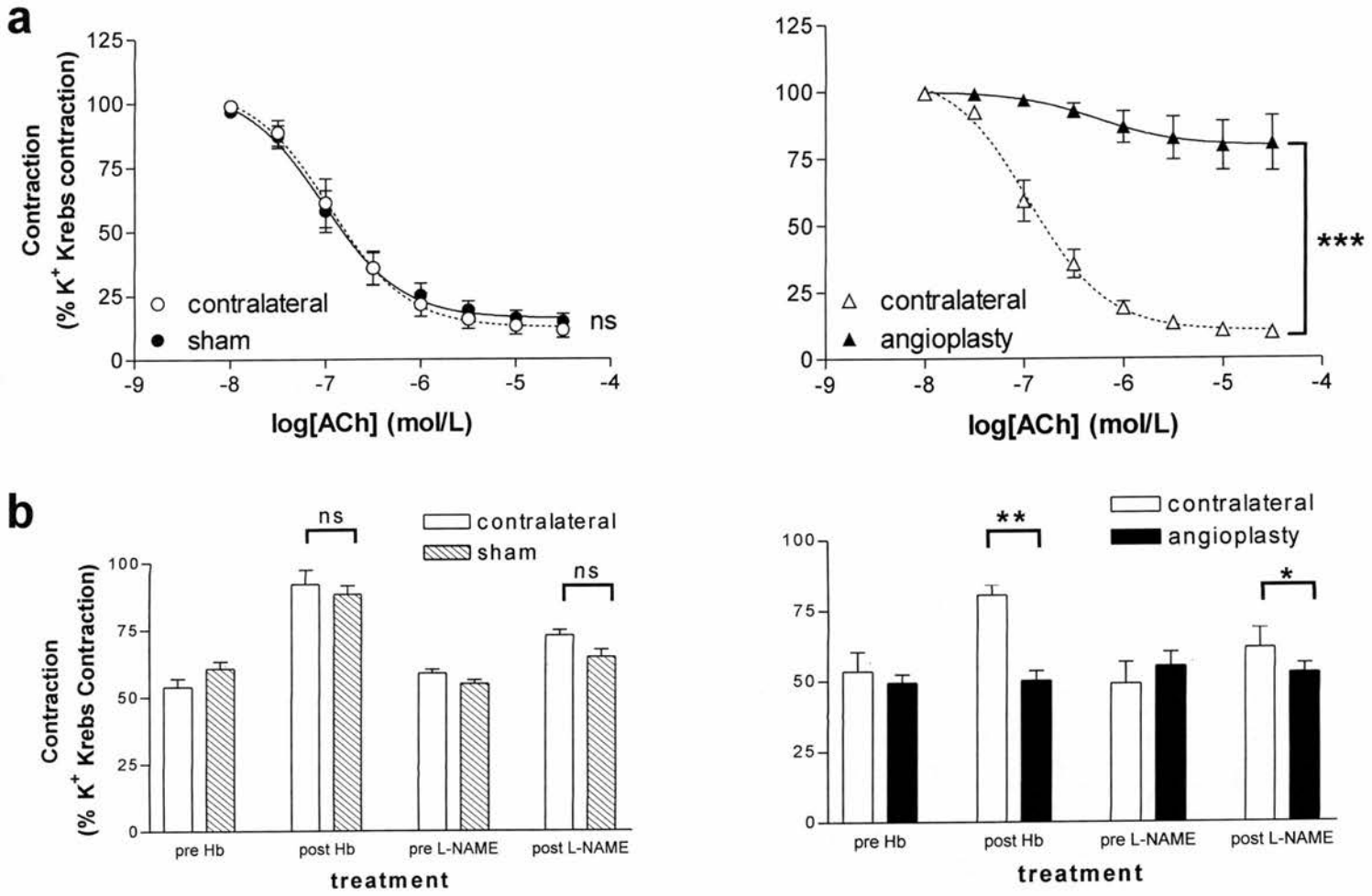


Figure 7.4 Vasodilator responses in carotid rings from sham-operated (filled circles/hatched columns), angioplastied (filled triangles/filled columns) and their corresponding contralateral (open symbols/open columns) vessels. (a) Response to ACh (0.01-30 μM). Rings are preconstricted with an EC_{80} does of PE (~3 μM). (b) Response to Hb (10 μM) and L-NAME (200 μM). Rings are preconstricted with an EC_{50} dose of PE (~1 μM). Changes in tension are expressed as % maximum response to high K^+ Krebs. Mean ± S.E.M. (n=6-7).

7.3.2 Effect of drug bolus on vessel function *ex vivo*

In both angioplastied and contralateral vessels, bolus administration of either NO donor immediately after angioplasty *in vivo* had no significant effect on responses to high K^+ Krebs (P=0.18 and P=0.08, for angioplastied and contralateral vessels respectively; 1-way unrelated ANOVA; n=5-6), PE (EC_{50}=0.6-1.5 μM; P=0.33 for both; 1-way unrelated ANOVA; n=5-6), ACh (EC_{50}=47-120 nM; P=0.14 for contralateral arteries; 1-way unrelated ANOVA; n=5-6), Hb (P=0.76 and P=0.88, respectively; 1-way unrelated ANOVA; n=5-6) or L-NAME (P=0.46 and P=0.35, respectively; 1-way unrelated ANOVA; n=5-6).

7.3.3 Effect of drug bolus on blood pressure

Before angioplasty, baseline systolic and diastolic BP was 61±4 and 44±3 mmHg (n=21) respectively, and heart rate (HR) was 220±9 bpm (n=17). Hep-Sal bolus (0.2 ml) had no effect on BP or HR (P>0.43 for all; paired t-test; n=9; Fig 7.5). GTN bolus (200 nmol) caused a significant transient reduction in both systolic BP (-31±6%) and diastolic BP (-30±6%; P=0.02 for both; paired t-test; n=6; Fig 7.5), whereas SNVP (200 nmol) had no significant effect on systolic BP (-7±3%) or diastolic BP (-6±3%, P=0.07 and P=0.08, respectively; n=6 for both; Fig 7. 5).

7.3.4 Effect of drug bolus on plasma catecholamines

Drug boluses had no effect on plasma adrenaline (pre - post; nM; Hep-Sal, 0.5±0.1-0.5±0.2; GTN, 0.9±0.3-0.7±0.3; SNVP, 0.7±0.2-0.5±0.2; n=3-4) or noradrenaline (pre - post; nM; Hep-Sal, 1.8±0.3-1.3±0.3; GTN, 2.1±0.3-1.6±0.4; SNVP, 2.1±0.5-2.5±0.8; n=3-4); see Fig 7.6. Dopamine was not detected in any of the samples.

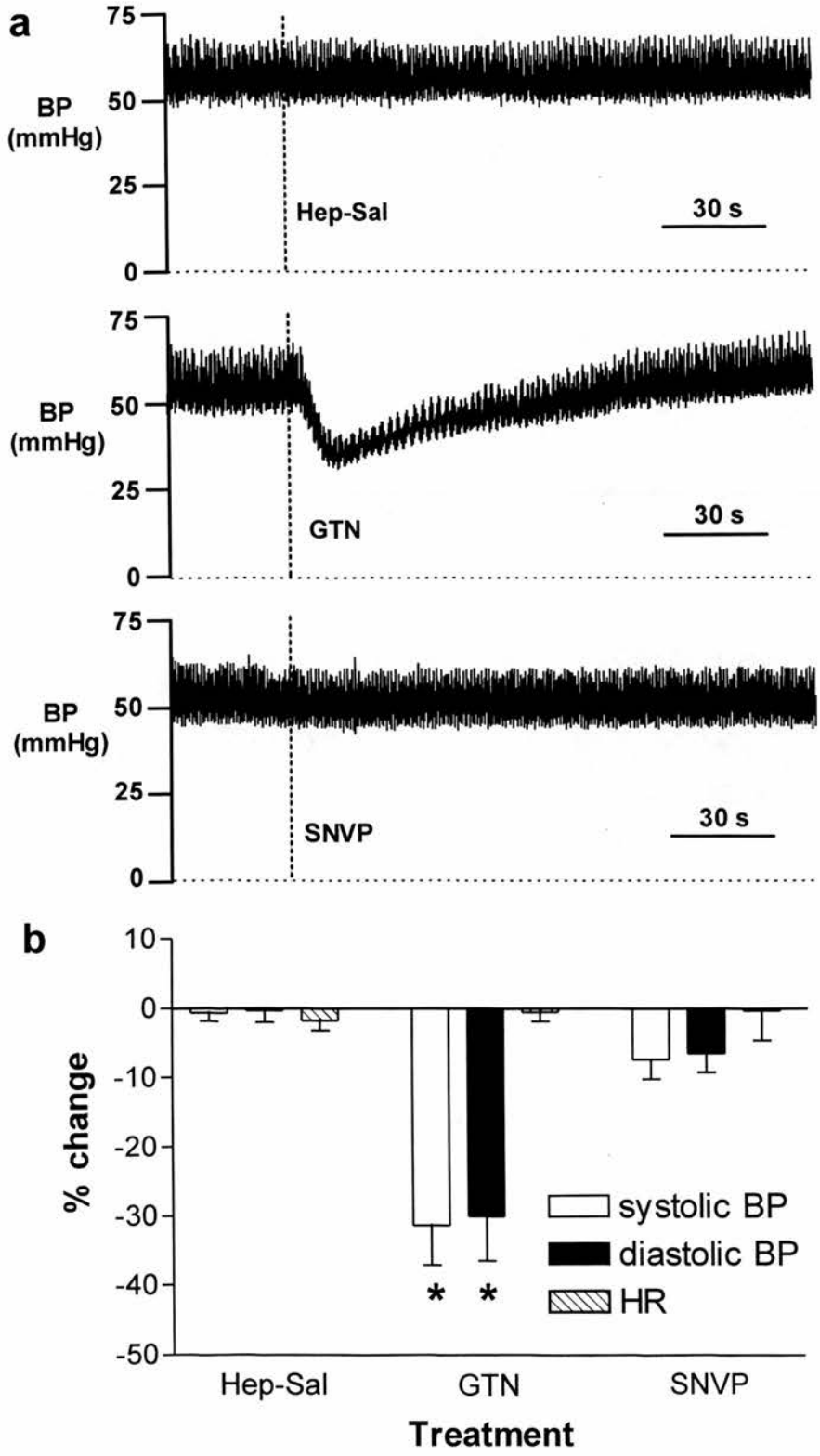


Figure 7.5 Changes in blood pressure induced by drug boluses. (a) Representative pressure recordings of the response to Hep-Sal (control), GTN and SNVP (both 200 nmoles). Vertical dotted line represents drug administration. (b) Effect of boluses (0.2 ml) of Hep-Sal, GTN and SNVP on systolic (open columns) and diastolic (filled columns) blood pressure and heart rate (hatched columns). Expressed as maximum change in BP or HR as a % of baseline. Mean±S.E.M. (n=6-9).

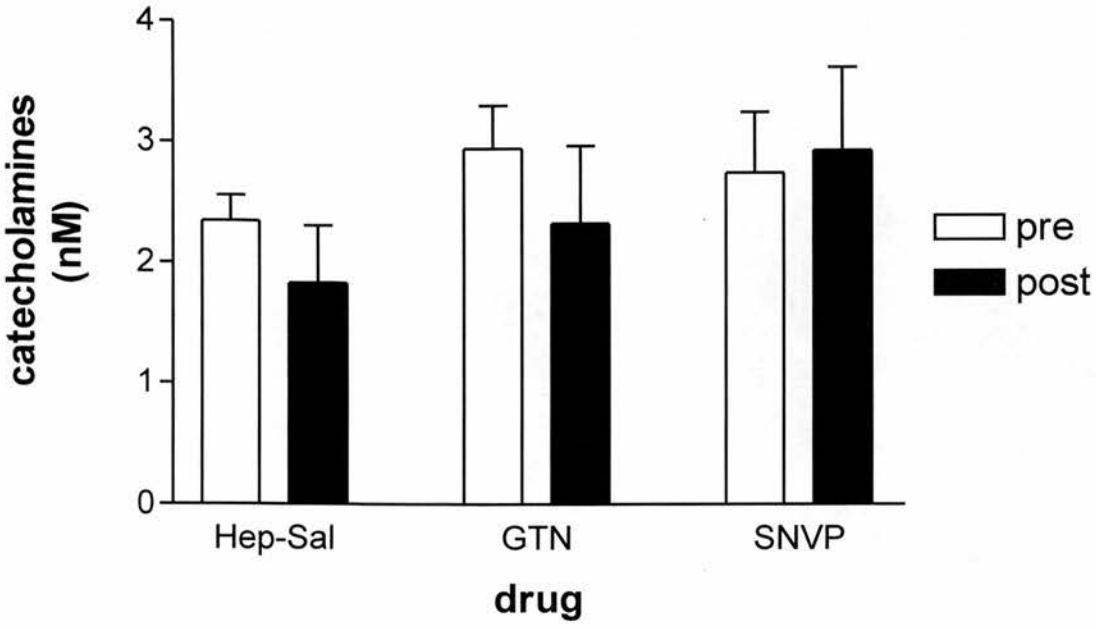


Figure 7.6 Effect of drug boluses on plasma catecholamines (noradrenaline and adrenaline). Open columns (before angioplasty and drug bolus), closed columns (after drug bolus). Mean±S.E.M. (n=3-4).

7.3.5 Effect of drug bolus on platelet aggregation

Following angioplasty with Hep-Sal, there was a 9.3±2.6 mV increase (~20%) in platelet aggregation to ADP (8 μM) compared to pre-angioplasty results (P=0.04; paired t-test; n=4; Fig 7.7a). In contrast, angioplasty rabbits that received GTN or SNVP, there was no increase in aggregability (P>0.24; paired t-test; n=5 for both; Fig 7.7a).

7.3.6 Radiolabelled platelet adhesion to carotid arteries

Platelet labelling efficiency was ~35%. Adhesion of radiolabelled platelets was standardised to account for vessel length (0.4-0.6 cm) and radioactivity in whole blood (9570±169 dpm/100 μl; n=20). In angioplastied vessels, there was an almost 20-fold increase in platelet adhesion (index=0.25±0.04; Fig 7.7b) compared to contralateral vessels (index=0.013±0.01 dpm). GTN did not significantly reduce platelet adhesion in angioplastied vessels in comparison to Hep-Sal (P=0.30; unpaired t-test; n=6 and 7, respectively), whereas SNVP reduced it by 62±7% (P=0.003; unpaired t-test; n=7; Fig 7.7b). The inhibitory effect was significantly greater than GTN (P=0.01; unpaired t-test; n=7 for SNVP and 6 for GTN).

7.3.7 Electron microscopy

Adhesion of platelets to the intimal surface of blood vessels was confirmed using scanning EM (Fig 7.8). In contralateral vessels, the luminal ridges can be seen with few cells adhering (Fig 7.8a). Following a Hep-Sal bolus, angioplastied vessels showed platelets covering the entire luminal surface (Fig 7.8b). Extended pseudopodia and connective strands can be seen to form a mesh between activated platelets. In angioplastied vessels treated with SNVP, the number of activated platelets adhering to

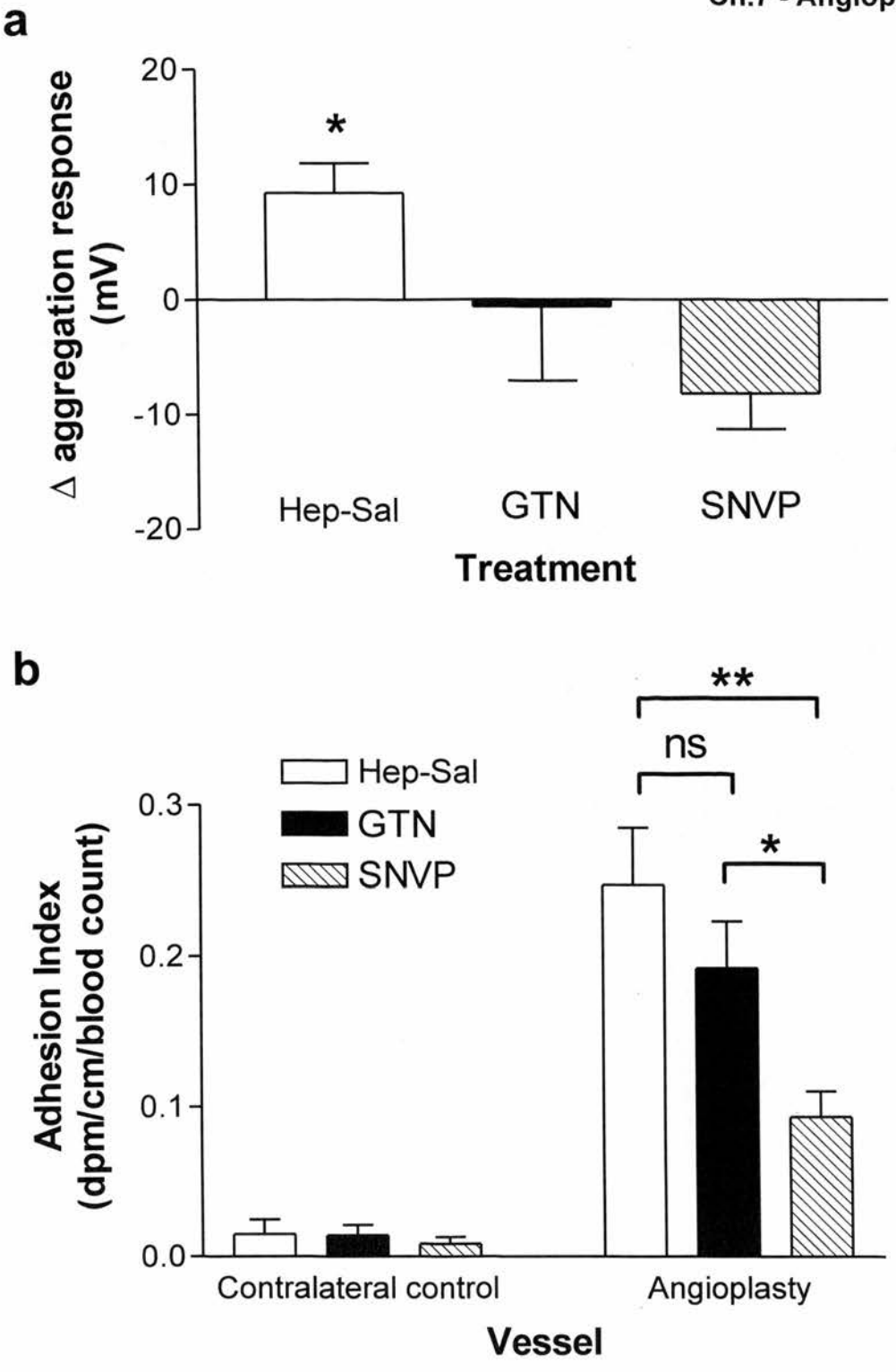


Figure 7.7 Effect of Hep-Sal (open columns), GTN (filled columns) and SNVP (hatched columns) on platelet activation. (a) *In vitro* platelet aggregation in response to ADP (8 μM). Responses expressed as change in aggregation after angioplasty and drug administration, compared to before the procedure. (b) Platelet adhesion to angioplastied and contralateral (uninjured) carotid arteries following drug treatment (0.2 ml; 200 nmoles). Radiolabelled-platelet adhesion is expressed as dpm, standardised to vessel length and whole blood radioactivity. Vessels were isolated and platelet adhesion measured 30 min after drug treatment. Mean±S.E.M. (n=4-7).

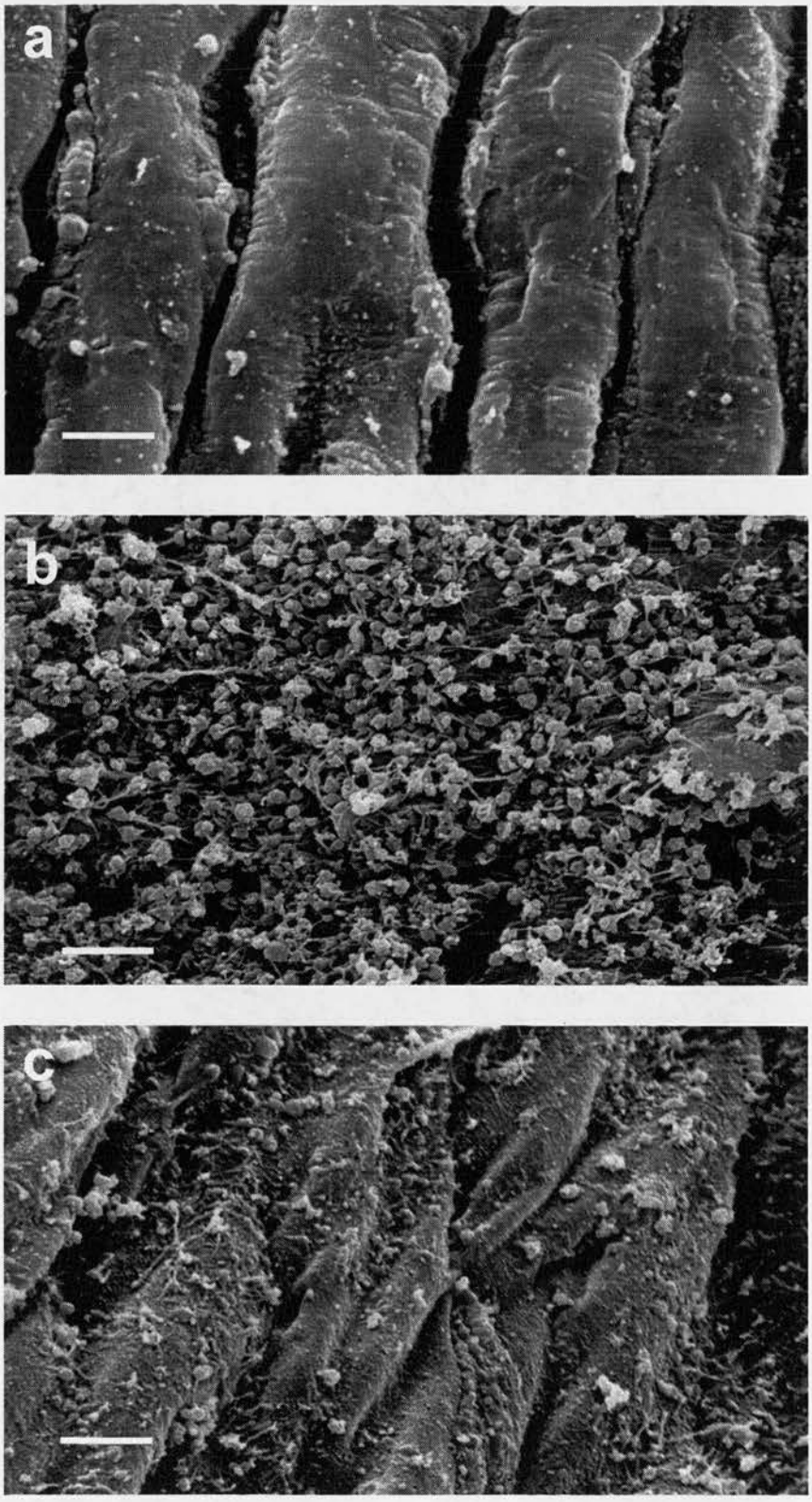


Figure 7.8 Representative scanning electron micrographs of the luminal surface of the carotid artery. (a) Contralateral (uninjured) artery, (b) angioplastied artery receiving Hep-Sal bolus (0.2 ml), (c) angioplastied artery receiving SNVP bolus (200 nmoles). Vessels were isolated and fixed 30 min after drug treatment. White bar represents 10 μm.

the luminal surface was substantially reduced (Fig 7.8c). The flattened, distended ridges of the angioplastied vessel can now be seen.

Transmission EM revealed platelets without cytoplasmic granules and pseudopodia extending over the intimal surface, confirming both adhesion and activation (Fig 7.9).

7.3.8 Histochemistry and immunohistochemistry

Haematoxylin and eosin staining demonstrated the presence of nucleated cells on the luminal surface of the internal elastic lamina of contralateral uninjured arteries (Fig 7.10a). In contrast, in angioplastied vessels, there were no nucleated cells present in the intima (Fig 7.10b).

There was intense GSL staining of nucleated cells on the luminal surface of contralateral uninjured arteries (Fig 7.11a). In contrast, in angioplastied vessels, there was only weak staining of the intima with no nucleated cells present (Fig 7.11b). Small areas stained more intensely and were granular in appearance, showing regions of the damaged intima with greater numbers of adherent blood cells.

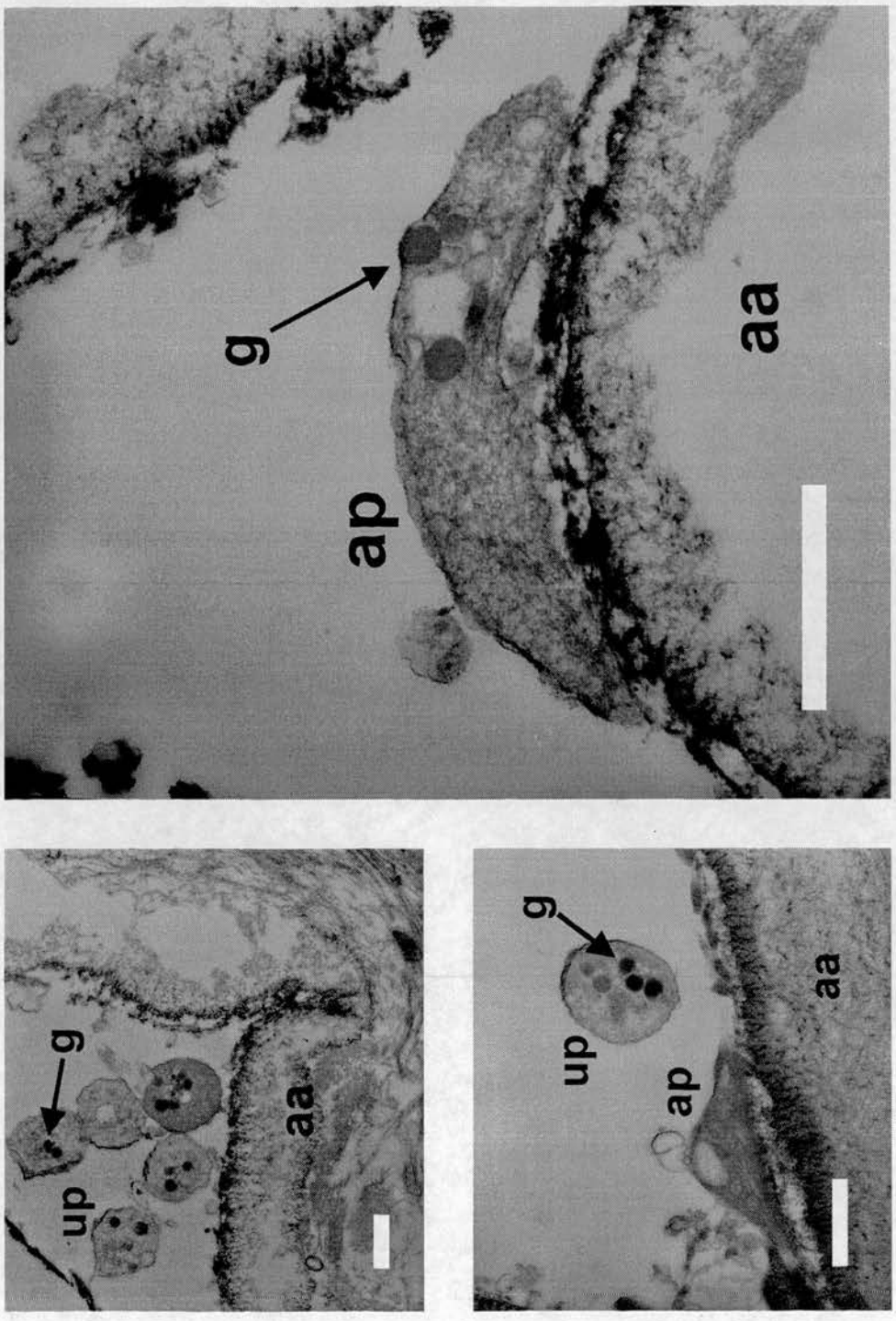


Figure 7.9 Representative transmission electron micrographs of platelets adhering to an angioplastied artery (aa). Adherent platelets (ap) have few cytoplasmic granules (g) compared to unattached platelets (up). Vessels were isolated and fixed 30 min after drug treatment. White bars represent 1 μm.

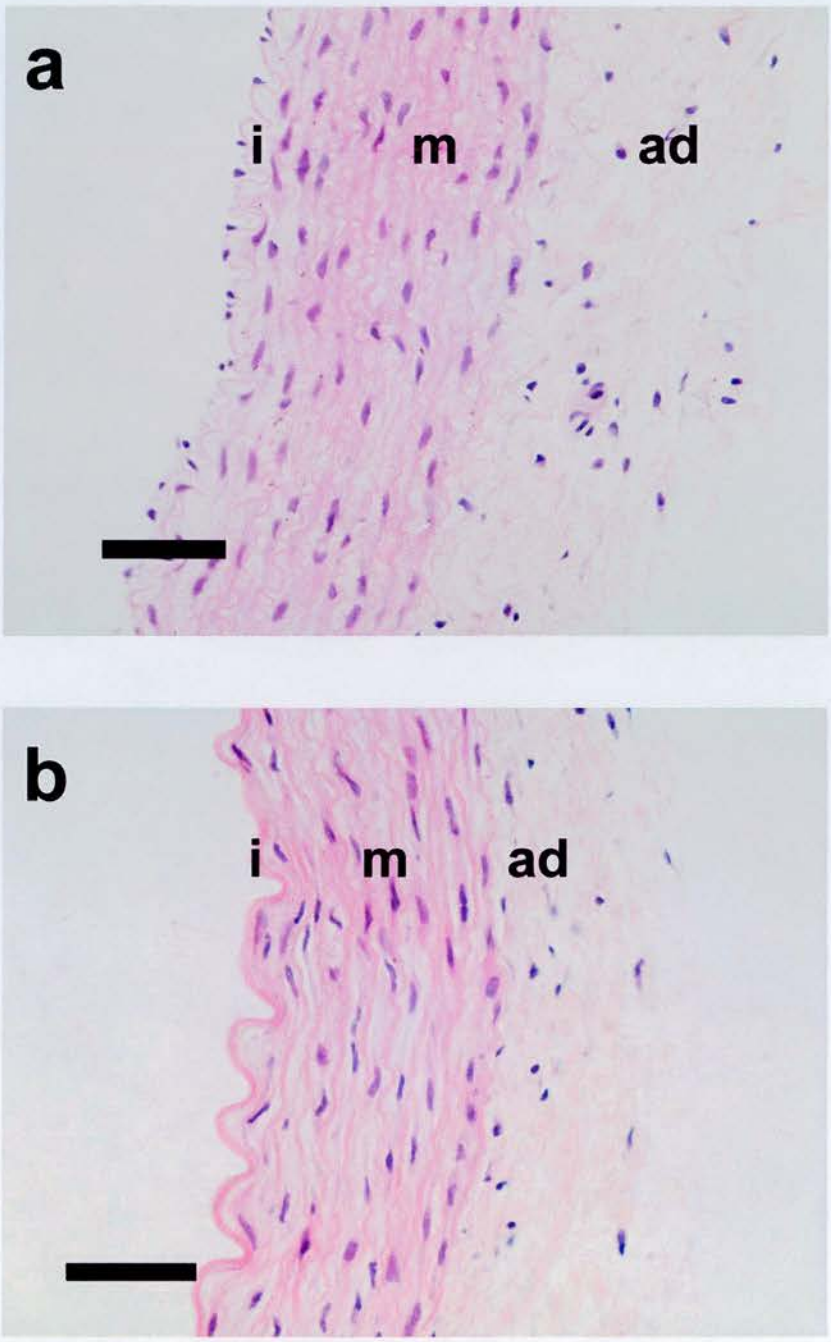


Figure 7.10 Representative images of sections of carotid arteries, with cell nuclei stained with haematoxylin (purple) and general tissue structure stained with eosin (pink). (a) Contralateral (uninjured) artery. (b) Angioplastied artery; note that there are no nucleated cells in the intimal layer (i). Media (m), adventitia (ad). Vessels were isolated and fixed 30 minutes after drug treatment. Black bar represents 50 μm.

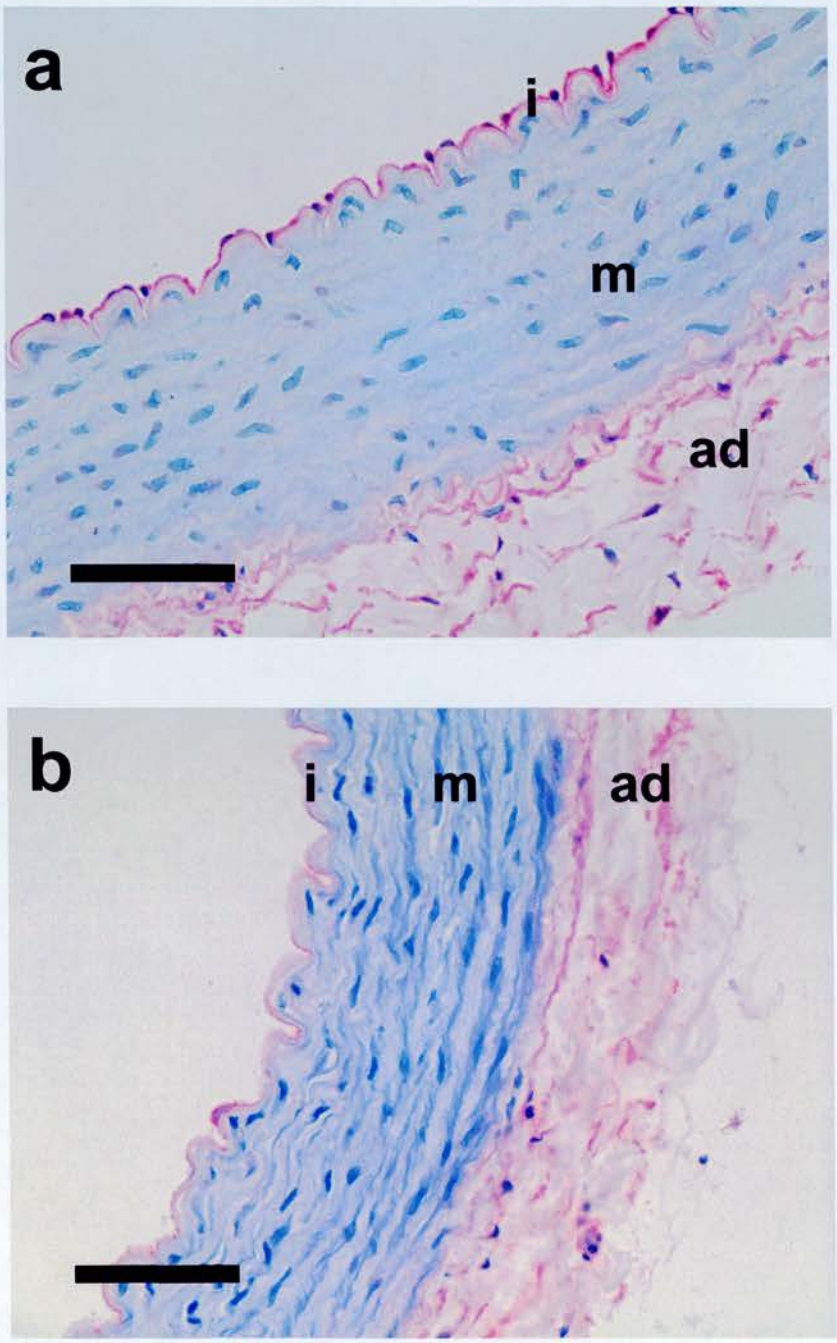


Figure 7.11 Representative images of sections of carotid arteries, showing immunoreactivity against GSL (red/pink staining). (a) Contralateral (uninjured) artery. There is intense red staining of the nucleated cells of the endothelium. (b) Angioplastied artery. There is only weak staining of the intimal layer (i). Media (m), adventitia (ad). Vessels were isolated and fixed 30 min after drug treatment. Counterstaining of nuclei with Harris' haematoxylin (purple/dark blue) and general tissue staining with methyl green (turquoise/light blue). Black bar represents 50 μm.

7.4 DISCUSSION

This study demonstrates that a novel S-nitrosothiol, SNVP, reduces adhesion of radiolabelled platelets to rabbit carotid arteries following angioplasty *in vivo*, without significantly affecting systemic BP. In contrast, equimolar GTN caused an undesirable decrease (~30%) in systemic BP, but failed to inhibit local platelet adhesion. Both NO donors prevented circulating platelet hyper-aggregability induced by angioplasty, suggesting that the added benefits of SNVP are due to prolonged antiplatelet action selectively at sites of endothelial cell damage.

7.4.1 Limitations of angioplasty and current therapies

The primary limitation of angioplasty is restenosis of treated arteries within six months of intervention. The underlying causes of restenosis have not be fully characterised, although cellular proliferation, migration and vascular remodeling are evident several days after injury (Wei Liu *et al.*, 1989; Lincoff *et al.*, 1994). The technique inevitably damages the vascular endothelium, and subsequent platelet adhesion is a key early event of restenosis (Friedman *et al.*, 1977; Fingerle *et al.*, 1989). Platelets form a monolayer on the exposed subendothelium and release the majority of their granules, containing chemotactic agents, vasoconstrictors and growth factors, within an hour after angioplasty (Clowes & Karnovsky, 1977; Groves *et al.*, 1979; Wei Liu *et al.*, 1989). Current anti-thrombotic therapies, including aspirin, clopidogrel and GPIIb/IIIa antagonists, have been shown to reduce platelet adhesion and intimal thickening following angioplasty (Topol & Serruys, 1998; Bult, 2000; Swanson *et al.*, 2001). However, maintaining sufficiently high local drug concentration over a number of hours can be problematic (Groves *et al.*, 1986; Lincoff *et al.*, 1994; Wolinsky, 1994). Recently, sirolimus (rapamycin)-eluting stents have been shown to be particularly

effective at inhibiting neointimal hyperplasia, (Sousa *et al.*, 2001; Suzuki *et al.*, 2001; Swanson *et al.*, 2001) but this approach does not have anti-platelet actions and the long-term effectiveness (> 1 yr) has yet to be established. Despite current advances in drug delivery catheters (Lincoff *et al.*, 1994) and deployment of stents, (Swanson *et al.*, 2001) endothelial cell damage and platelet activation still remain a significant problem, highlighting the need for additional therapies which counteract the multiple factors involved in the restenotic process (Bult, 2000; Janero & Ewing, 2000).

7.4.2 Role of conventional NO donors

Delivery of NO is an attractive alternative to conventional anti-thrombotic agents, because NO exhibits a range of beneficial actions, including vasodilatation, regulation of inflammatory cell function, inhibition of smooth muscle cell mitogenesis and inhibition of platelet activation (Janero & Ewing, 2000; Megson, 2000; Vural & Bayazit, 2001). Indeed, NO donors inhibit platelet activation following angioplasty (Lam *et al.*, 1988; Groves *et al.*, 1993; Langford *et al.*, 1994). The most common clinically used NO donors are the organic nitrates, such as GTN, although the selectivity profile of traditional nitrates (veins>arteries>platelets; MacAllister *et al.*, 1995) is unfavourable. The current results are in keeping with the recognised selectivity profile of GTN; it caused a profound systemic hypotension (~30 %), demonstrating that vasoactive concentrations of GTN were being used that may induce unwanted complications in patients undergoing angioplasty. Baroreceptors unloading in response to hypotension could activate the sympathetic nervous system, with release of catecholamines. In turn, this may enhance platelet activation in response to agonists (Haslam & Taylor, 1971) and could counterbalance any direct inhibitory effects of GTN on platelet adhesion. However, plasma catecholamines and HR were not affected by the administration of NO donors, therefore, baroreceptor unloading did not importantly influence the actions of GTN.

GTN is a poor inhibitor of platelet aggregation *in vitro*, (Megson & Webb, 2000) possibly because PRP lacks the factors necessary for its biotransformation to active NO (Weber *et al.*, 1996). *In vivo*, GTN infusion can inhibit platelet activation, presumably via vascular tissue-mediated biotransformation of GTN to NO (Folts *et al.*, 1991). GTN reduced platelet hyperaggregability *ex vivo*, however, it failed to prevent adhesion of radiolabelled platelets to the intimal surface of angioplastied carotid arteries. These results suggest that bolus GTN can influence activation of circulating platelets, but its short-term effects are not capable of preventing adhesion to the exposed subendothelial surface (Lam *et al.*, 1988). Chronic administration is, therefore, necessary for GTN to show benefit, but is limited by tolerance (Parker & Fung, 1984; Abrams *et al.*, 1998). Similarly, other drugs, such as aspirin, have also been shown to prevent platelet hyper-responsiveness without reducing platelet adhesion (Clowes & Karnovsky, 1977).

7.4.3 Potential of S-nitrosothiols

S-Nitrosothiols exhibit a number of properties that might prove advantageous in angioplasty. They show greater selectivity for arteries than veins (MacAllister *et al.*, 1995) and, unlike organic nitrates, are also powerful inhibitors of platelet aggregation both *in vitro* (Sogo *et al.*, 2000) and *in vivo* (De Belder *et al.*, 1994). S-nitrosothiols can generate sufficient NO in PRP to inhibit platelet aggregation (Sogo *et al.*, 2000). It has previously been shown that SNVP causes sustained NO-mediated vasodilatation selectively in blood vessels with damaged endothelium (Megson *et al.*, 1999). In addition, other novel lipophilic S-nitrosothiols produce sustained vasodilatation in human arteries and veins both *ex vivo* (Sogo *et al.*, 2000) and *in vivo*, (Sogo *et al.*, 2000) which may potentially be of great benefit in preventing vasospasm in angioplastied arteries. This evidence strongly suggests that SNVP is able to

specifically target areas of endothelial damage, a feature that is not shared by conventional NO donors.

Equimolar concentrations of SNVP were compared with GTN, because both compounds release 1 molar equivalent of NO (Bennett *et al.*, 1989; Megson *et al.*, 1999). In contrast to GTN, SNVP had minimal effects on systemic BP, but caused a >60% reduction in platelet adhesion to angioplastied carotid arteries. Both SNVP and GTN inhibited *in vitro* platelet aggregation in response to ADP, however, only SNVP inhibited platelet adhesion following angioplasty. Here, SNVP was administered as a concentrated bolus immediately upstream of the angioplastied region (Fig 7.1). Previous results (Megson *et al.*, 1999) are consistent with the hypothesis that SNVP is retained in the exposed subendothelial layers, where it decomposes slowly, generating sufficient NO to inhibit platelet adhesion locally. S-nitrosated albumin appears to act in a similar fashion (Marks *et al.*, 1995). The inhibitory effect of SNVP on platelet adhesion suggests that this compound may have therapeutic potential in the prevention of acute thrombosis at the site of angioplasty, possibly from a single administration.

Novel lipophilic S-nitrosothiols may also be of therapeutic benefit in preventing the progression of atherosclerosis. Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques (Mann & Young, 1994; Davies, 2000). The prolonged presence of residual thrombus over an eroded plaque promotes plaque expansion and destabilisation. By targeting delivery of NO to areas of erosion, novel lipophilic S-nitrosothiols could reduce thrombus deposition and, subsequently, plaque progression. Although spectulative, this opens the question of whether novel S-nitrosothiols have a role in the prevention of atherosclerotic cardiovascular disease.

7.4.4 Study limitations

Damaged vessels lose their hypereactivity to platelets within 8 h after injury, despite incomplete endothelial regrowth (Dewanjee *et al.*, 1984; Groves *et al.*, 1986). From the present study, it is unknown whether the antiplatelet effects of SNVP persist for >30 min after angioplasty. In endothelium-denuded rat femoral arteries, up to 75% of the vasodilatation to another lipophilic S-nitrosothiol, RIG200, is still present at 4 h, (Megson *et al.*, 1997) emphasising the long-acting nature of these compounds. This complementary vasodilator effect would be beneficial in limiting vasospasm following angioplasty and perhaps preclude the need for stenting. However, the results of functional experiments in the current study failed to confirm a prolonged vasodilator effect in rings from angioplastied carotid arteries treated with SNVP. There was no difference in the maximum contraction to KCl or PE between vessels that received different NO donors and no evidence from Hb experiments of NO-mediated, sustained vasodilatation in SNVP-treated vessels. However, these results do not preclude the possibility that sufficient S-nitrosothiol remains in the vessel to inhibit platelet adhesion, but not to cause vasodilatation. Alternatively, the setup time and processing of the isolated carotid ring experiments may reduce the concentrations of drug retained in the tissue. Further experiments are needed to investigate the mechanism and duration of action of drugs like SNVP, and will help to establish their therapeutic potential in the prevention of restenosis.

7.4.5 Summary

The lipophilic S-nitrosothiol, SNVP, markedly reduces platelet adhesion to damaged arteries, following angioplasty, from a single bolus administration that does not cause systemic hypotension. The long-lasting antiplatelet effects could be of therapeutic potential in the prevention of restenosis and the progression of atherosclerosis, either alone or as an adjunct to current thrombolytic therapies and stenting.

Chapter 8

General Discussion

8. GENERAL DISCUSSION

8.1 INTRODUCTION

The vascular endothelium has many functions, one of the most important of which is the synthesis of vasoactive substances, including NO. The bioavailability of NO is diminished in a number of cardiovascular diseases, and subsequently, the delivery of exogenous NO is an attractive therapeutic option in the treatment of these conditions (Vallance & Chan, 2001; Ignarro *et al.*, 2002).

Conventional nitric oxide donor drugs are used in suprisingly few clinical applications. One explanation for this is that established NO donors, such as organic nitrates and SNP have limitations, especially in that they cannot be used continuously long-term due to the development of tolerance or toxicity of by-products, respectively (Megson, 2000; Ignarro *et al.*, 2002). S-nitrosothiols are an attractive alternative, as they are unlikely to generate toxic by-products and do not induce tolerance. Recently, Megson *et al* described several lipophilic S-nitrosothiols, including RIG200 (Megson *et al.*, 1997) and SNVP (Megson *et al.*, 1999), that cause sustained vasodilatation in endothelium-denuded arteries. Therefore, these compounds may be able to selectively deliver NO to areas of endothelial damage and thus limit systemic side-effects.

This thesis investigates the mechanism of action of these novel lipophilic S-nitrosothiols in comparison to established NO donors, using isolated rat femoral arteries in an *in vitro* perfusion system. Compounds can be given as a bolus or in the vessel perfusate and, therefore, mimic *in vivo* administration by injection or infusion, through selective delivery to the vessel lumen. In addition, an *in vivo* rabbit model is used to investigate the potential of lipophilic S-nitrosothiols in the prevention of platelet adhesion to areas of endothelial damage caused by balloon angioplasty.

8.2 MECHANISM OF THE VASODILATOR ACTION OF S-NITROSOTHIOLS IN COMPARISON TO OTHER NO DONORS

Nitric oxide initiates vasodilatation by binding to the haem moiety of sGC, catalysing the synthesis of cGMP in VSMCs. cGMP stimulates PKG, which ultimately reduces cytosolic calcium and inhibits the contraction of vascular smooth muscle, causing vasodilatation (Waldman & Murad, 1987; Moncada *et al.*, 1991; Carvajal *et al.*, 2000). However, NO and several NO donors have been shown to have sGC-independent actions at high concentrations (Gordge *et al.*, 1998; Homer & Wanstall, 2000; Sogo *et al.*, 2000; Wanstall *et al.*, 2001). In Chapter 3, the vasodilator effect of NO donors is investigated in isolated arteries that have been treated with the highly specific sGC inhibitor, ODQ. The results show that NO donor drugs that release NO at an intracellular site cause vasodilatation that is abolished by ODQ. However, compounds that release NO extracellularly, particularly SPER/NO, produce an NO-mediated vasodilatation that is partially resistant to ODQ. Therefore, it is likely that the proximity and rapid binding of intracellular NO to sGC competes with the reaction of NO with other cytosolic components that may mediate sGC-independent effects. However, in the extracellular space, high concentrations of NO can react with other factors, such as molecular oxygen (Ford *et al.*, 1993; Kharitonov *et al.*, 1995; Keshive *et al.*, 1996) or superoxide (Freedman & Crapo, 1982; Mayer *et al.*, 1998; Vinten-Johansen, 2000). sGC-independent vasodilatation is inhibited by superoxide generators, suggesting that NO instead reacts with molecular oxygen to produce higher nitrogen oxides. Subsequently, these products are able to nitrosate membrane associated SH-containing molecules, leading to sGC-independent vasodilatation. In contrast, peroxynitrite, the reaction product with superoxide, is unlikely to mediate sGC-independent

vasodilatation. Unlike SPER/NO, the S-nitrosothiols, GSNO and SNVP, cause vasodilatation that is largely sGC-dependent. Only very high concentrations of S-nitrosothiols were able to elicit vasodilatation with a small sGC-independent component. Consequently, this sGC-independent vasodilatation is unlikely to be of physiological significance in terms of vascular tone, although sGC-independent actions may be of importance in mediating their potent antiplatelet actions (Sogo *et al.*, 2000). It is possible that the sizeable sGC-independent actions of S-nitrosothiols accounts for the platelet selectivity of these compounds (De Belder *et al.*, 1994).

Chapter 4 investigates the role of thiols in the vasodilatation of NO donors, using agents that block or deplete free thiols and the SH groups of thiol-containing proteins. The results highlight the importance of thiols in the vasodilator action of all the NO donors, particularly GTN. Additionally, thiol depletion also produced a small inhibition of the vasodilatation caused by the NO:sGC-independent vasodilator, ISP. GSH is the most common intracellular thiol and a highly important cellular antioxidant (Meister, 1994; Griffith, 1999). Subsequently, depletion of GSH leads to increased oxidative stress. As shown in Chapter 3, enhanced superoxide levels when antioxidant systems are compromised by Cu/Zn-SOD inhibition, lead to a marked attenuation of the vasodilator actions of NO donors, and a small attenuation of responses to ISP. Global modification of endogenous thiols using EA causes further non-specific inhibition, highlighting the critical role of SH groups in vasodilatation. Candidate regulatory molecules include the SH groups of sGC (Braughler, 1983; Kamasaki *et al.*, 1986), AC (Guillon *et al.*, 1981), receptor coupling proteins (Mukherjee & Mukherjee, 1981; Suen *et al.*, 1982) and myosin (Kubberod *et al.*, 1974; Stamler *et al.*, 1992).

Importantly, NO donors were differentially affected by thiol depletion. S-nitrosothiols and SNP were relatively less susceptible to thiol depletion than GTN. This suggests that thiols are not an essential requirement for the release of NO from these compounds. Additionally, it could be suggested that these drugs are less

susceptible to oxidative stress. However, Chapter 3 shows that superoxide inhibits S-nitrosothiol-induced vasodilatation to a greater extent than that induced by GTN; an NO donor which is relatively resistant to superoxide generation, but particularly susceptible to thiol depletion. It is generally accepted that thiols *alone* are not capable of metabolising organic nitrates (Schroder, 1985; Megson, 2000) and it has been proposed that enzymatic pathways, such as cP450 enzymes, mediate the bioconversion of nitrates (Schroder & Schror, 1990; Yeates, 1992; Bennett *et al.*, 1994). However, the susceptibility of GTN to thiol depletion suggests that the enzyme mediating the biotransformation of organic nitrates may require thiols to act as co-factors. This could explain why specific thiols, such as cysteine, seem to be required for the activation of sGC by GTN (Ignarro *et al.*, 1981).

The identification of the enzyme systems that metabolise organic nitrates and other NO donors will undoubtedly shed some light on the mechanism of tolerance induction. Tolerance was investigated in Chapter 5 by perfusing isolated arteries for 20 h with NO donors. Cross-tolerance could then be investigated using bolus administration of NO donors in vessels after long-term perfusion with GTN. The results show that GTN induces tolerance rapidly; within ~2 h of continuous perfusion. In contrast, S-nitrosothiols do not induce self-tolerance and remain fully active in nitrate-tolerant vessels. Recently, two theories to explain the underlying cause of tolerance have received much attention (Parker & Gori, 2001). The first of these suggest that prolonged administration of organic nitrates leads to generation of superoxide, that scavenges nitrate-derived NO (Munzel *et al.*, 1995). The current results dispute this hypothesis, as S-nitrosothiols are fully active in nitrate-tolerant vessels, yet far more susceptible to superoxide than GTN (Chapter 3). Secondly, it has been suggested that prolonged GTN desensitises sGC (Axelsson & Andersson, 1983; Waldman *et al.*, 1986) or upregulates PDEs (Axelsson & Andersson, 1983; Kim *et al.*, 2001). Again, these theories are contradicted by the finding that S-nitrosothiols are resistant to tolerance, yet cause a vasodilatation that is almost entirely mediated by

sGC. Overall, it is concluded that tolerance induction is a symptom of dysfunction of the metabolic process required to release NO from nitroxy ester groups, perhaps through downregulation of the enzyme that biotransforms organic nitrates (Bennett *et al.*, 1994).

From a pragmatic standpoint, it is important to note that, similarly to other S-nitrosothiols (Kowaluk *et al.*, 1987; Bauer & Fung, 1991; Shaffer *et al.*, 1992), the novel lipophilic S-nitrosothiols do not induce tolerance. These results demonstrate that the structural adaptations made to RIG200 and SNVP do not affect the resistance to tolerance of these compounds, and implies a further therapeutic benefit over organic nitrates in cardiovascular conditions where long-term continuous therapy is required.

A summary of the speculated mechanisms of action of NO donors used in the above experiments is shown in Fig 8.1.

8.3 SUSTAINED ACTIONS OF LIPOPHILIC S-NITROSOTHIOLS IN ARTERIES WITH A DAMAGED ENDOTHELIUM

8.3.1 Role of lipophilicity in the vasodilatation in response to glucosamine-linked S-nitrosothiols

Chapter 5 focuses on the sustained vasodilator actions of lipophilic S-nitrosothiols in denuded femoral arteries. The experiments employed use an *in vitro* perfusion system in order to facilitate delivery of S-nitrosothiols as boluses, selectively to the vessel lumen. This negates the possibility that lipophilic S-nitrosothiols could adhere to the adventitial surface, irrespective of endothelial integrity. Traditional organ bath systems

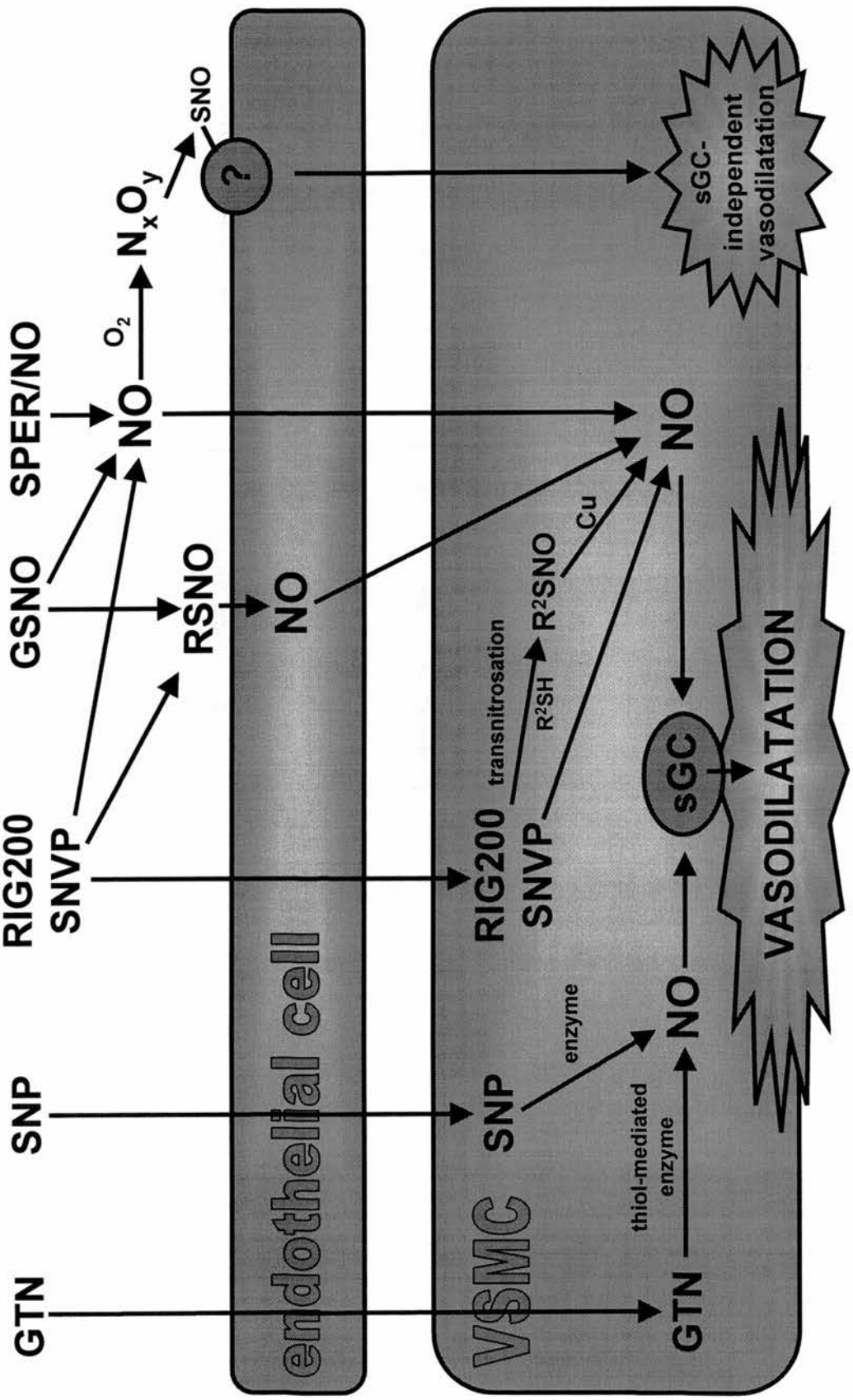


Figure 8.1 Speculative hypotheses of the mechanism of activation of NO donors in endothelium-intact arteries.

bathe vessels in drugs and therefore would expose the adventitial surface to lipophilic compounds.

Previously, Megson *et al.* demonstrated that sustained vasodilatation is correlated with the lipophilicity of N-substituted analogues of SNAP (Megson *et al.*, 1999). Chapter 5 considered the question of whether lipophilicity is important in sustained vasodilatation produced by glucosamine-linked SNAP analogues. Glyco-SNAP contains an non-acetylated glucosamine group and subsequently is highly water soluble (Ramirez *et al.*, 1996) in comparison to RIG200, where the hydroxyl groups of the glucosamine moiety are acetylated.

The results from experiments using bolus injections into the perfusate show that RIG200 produced a marked, substantially NO mediated, sustained vasodilatation (~50-65 %) 1 h after bolus administration to endothelium-denuded vessels. A small Hb-resistant, non-specific vasodilatation remained in both endothelium-intact and denuded vessels. However, glyco-SNAP was found to cause a small sustained effect, irrespective of endothelial integrity. Interestingly, the sustained effect in response to glyco-SNAP was reversed by Hb and is clearly due to a different mechanism that the effect seen with RIG200 in endothelium-denuded vessels. It is likely that the difference reflects the degree of penetration of the two compounds into the vascular tissue. RIG200 may penetrate deeper into the tissue, or even access cells, whereas small concentrations of glyco-SNAP may be retained in superficial water-soluble compartments that are accessible by Hb.

8.3.2 Effect of lipophilic S-nitrosothiols following balloon angioplasty

Previous studies had demonstrated that the lipophilic S-nitrosothiols were capable of producing sustained vasodilator effects in human arteries and veins *in vitro* (Sogo *et al.*, 2000) and *in vivo* (Sogo *et al.*, 2000). However, *in vivo* studies did not

investigate the systemic effects of bolus administration of high concentrations of lipophilic S-nitrosothiols. Additionally, it remained to be established whether lipophilic S-nitrosothiols have prolonged antiplatelet actions at sites of endothelial damage *in vivo*. In Chapter 7, the antiplatelet effects of SNVP were investigated in an *in vivo* rabbit model of balloon angioplasty. Angioplasty itself (Hep-Sal control) increased the responsiveness of circulating platelets *ex vivo*. Additionally, angioplasty led to a large increase in the number of radiolabelled platelets adhering to arteries that had been denuded of endothelium by the procedure. Bolus administration of the conventional NO donor, GTN, produced a large reduction in systemic blood pressure (~30%) and prevented the increase in platelet aggregability. Despite having anti-platelet actions, presumably via tissue-mediated decomposition to NO (Folts *et al.*, 1991), GTN was unable to reduce platelet adhesion to the angioplastied region. In contrast, equimolar SNVP prevented the hyperaggregability of platelets and also caused a >60% reduction in platelets adhering to the angioplastied artery. Moreover, SNVP did not cause an undesireable fall in systemic blood pressure. Both NO donors prevented the hyperaggregability of circulating platelets, but had different abilities to inhibit platelet adhesion. Consequently, the inhibitory effect of SNVP on platelet adhesion is likely to be due to the sustained actions of this class of compound at sites of endothelial denudation. Overall, the results support the hypothesis that sufficient SNVP is retained at the site of angioplasty to inhibit platelet adhesion, but not to produce systemic vasodilatation. Platelet adhesion, and release of their cytoplasmic granules, is a key event in the initiation of restenosis, the major limitation of angioplasty (Friedman *et al.*, 1977; Goldberg & Stemerman, 1980; Fingerle *et al.*, 1989). Subsequently, the sustained anti-platelet actions of lipophilic S-nitrosothiols may be of therapeutic benefit in reducing thrombosis and restenosis following balloon angioplasty.

8.4 FUTURE DIRECTIONS AND FURTHER STUDIES

Despite extensive research over the last decade, the mechanism of action of NO donors is still poorly understood. That said, many misconceptions about compounds acting via the NO pathway are being disregarded and it is slowly becoming recognised that different classes of NO-releasing compounds act in very different ways. One of the most important milestones to be reached is the identification of the enzyme system(s) that release NO from donors *in vivo*. In particular, the identification of the thiol-dependent enzyme mediating the biotransformation of organic nitrates will help clarify three decades of contradictory literature and open up a new avenue of therapeutic alternatives to avoid the induction of tolerance.

As this thesis highlights, S-nitrosothiols are rapidly rising to attention as therapeutic alternatives to existing NO donor drugs. Subsequently, it is crucial that the endogenous factor(s) responsible for their metabolism is identified. Cell surface thiol groups are an obvious candidate for the factor initiating NO release and transfer into the cell (Zai *et al.*, 1999), accounting for the equipotent vasodilator actions of chemically diverse S-nitrosothiols (Kowaluk & Fung, 1990; Mathews & Kerr, 1993). However, existing pharmacological agents that block extracellular thiols, such as DTNB, may not access biologically relevant SH groups (Jiang *et al.*, 1999), making it difficult to draw accurate conclusions as to the involvement of cell surface thiols. An in-depth study using structurally modified cell impermeable thiol inhibitors may clarify this point. Additionally, the characterisation of the membrane bound S-nitrosothiol-metabolising protein/enzyme identified by Kowaluk *et al* (Kowaluk & Fung, 1990), offers the prospect of developing antisense to this protein, for use as a specific inhibitor of this step of the NO pathway, in a similar way to the antisense developed against csPDI (Zai *et al.*, 1999).

The ultimate aim of the pharmacologist is to develop drugs that are specific to certain regions of the body. At present, many NO donors show some degree of selectivity for large arteries, resistance beds, veins or platelets. Often though, the limited delivery methods require administration of systemic concentrations, overcoming regional selectivity. Headway is being made in the targeted delivery of NO, and already there are reports of NONOates (Saavedra *et al.*, 1997; Saavedra *et al.*, 1999; Saavedra *et al.*, 2000; Tang *et al.*, 2001; Wu *et al.*, 2001) and S-nitrosothiols (Al-Sa'doni *et al.*, 2000; Cantuaria *et al.*, 2000; Babich & Zuckerbraun, 2001; Hou *et al.*, 2001) that can only generate NO following cleavage by cell-specific factors, such as those within tumour cells. However, until recently there have been no reports of NO donors that specifically donate NO at regions where the bioavailability of endogenous NO is impaired. This thesis focuses on the mechanism of S-nitrosothiols that have selective vasodilator actions in endothelium-denuded arteries. It is hypothesised that these compounds are selectively retained at areas of endothelial damage (Megson *et al.*, 1997; Megson *et al.*, 1999). A similar proposal was offered for the anti-platelet effects of S-nitroso-Alb in arteries damaged by balloon angioplasty, but the effect of SNO-Alb on systemic BP was not measured (Marks *et al.*, 1995). Although a satisfactory alternative explanation for the sustained actions of these compounds has not been suggested, the 'retention hypothesis' still requires confirmation with radiolabelled S-nitrosothiols.

The lipophilic S-nitrosothiol, SNVP, was particularly effective in reducing platelet adhesion to angioplastied carotid arteries in comparison to GTN. However, GTN is relatively selective for veins over arteries (MacAllister *et al.*, 1995) and, therefore, the effectiveness of SNVP may have been due to its preferential metabolism within arteries, rather than its sustained effects. Now that a suitable model of platelet adhesion following angioplasty has been established, lipophilic S-nitrosothiols need to be compared to other arterioselective compounds. The parent S-nitrosothiol, SNAP, would be ideal for this purpose. Additionally, the more important consideration is to

look at the effectiveness of lipophilic S-nitrosothiols to inhibit platelet adhesion at time points longer than 30 min after angioplasty. It remains to be clarified if sufficient SNVP is retained in damaged blood vessels to prevent platelet activation until the injured vessel surface loses its reactivity to platelets, suggested to be ~8 h (Dewanjee *et al.*, 1984; Groves *et al.*, 1986). Furthermore, the consequences of this degree of platelet inhibition on restenosis need to be tested in a recovery model of angioplasty, such as the porcine coronary angioplasty model.

8.5 CLINICAL IMPLICATIONS

Novel S-nitrosothiols have a range of potential advantages over conventional NO donors and other therapies used for the treatment of cardiovascular conditions. These are summarised below:

- NO-releasing compounds have many beneficial actions in the cardiovascular system, including vasodilatation, inhibition of platelet activation, aggregation and adhesion, inhibition of inflammatory cells and inhibition of VSMC proliferation and migration.
- S-Nitrosothiols, such as SNO-Cys, mimic the actions of endogenous EDRF.
- S-Nitrosothiols are likely to be more easy to titrate than SNP.
- Existing S-nitrosothiols are unlikely to generate toxic by-products, and it is possible that thiol by-products may have antioxidant actions.
- S-Nitrosothiols can be used to selectivity dilate arteries over veins.
- S-Nitrosothiols show selectivity towards platelets over blood vessels.
- S-Nitrosothiols do not have strict metabolic requirements to release NO, in comparison to drugs like the organic nitrates

- S-Nitrosothiols do not induce self-tolerance and remain effective in nitrate-tolerant blood vessels
- S-Nitrosothiols may directly regulate protein function without the release of free NO (transnitrosation of SH groups).
- S-Nitrosothiols might protect NO against scavenging by oxygen free radicals.
- Novel lipophilic S-nitrosothiols are less susceptible to breakdown by trace metal ions found in physiological solutions, in comparison to conventional S-nitrosothiols.
- Lipophilic S-nitrosothiols can cause sustained vasodilatation at areas of endothelial damage.
- Lipophilic S-nitrosothiols prevent platelet adhesion to areas of endothelial damage *in vivo*.

Subsequently, S-nitrosothiols have show great potential in the treatment of cardiovascular disease. In contrast to the common used organic nitrates, S-nitrosothiols do not induce tolerance with continuous use. Importantly, the structural modifications made to the thiol groups to provide stability and lipophilicity, do not affect their tolerance profile. Therefore, these compounds could be used in the treatment of cardiovascular conditions where long-term therapy may be required, such as chronic angina.

A particularly attractive feature of these novel compounds is their sustained actions at sites of endothelial damage. As demonstrated in Chapter 7, lipophilic S-nitrosothiols have a use in the prevention of thrombosis following angioplasty. Additionally, the multifaceted nature of NO may also provide benefits by preventing vasospasm and restenosis through vasodilatation and inhibition of inflammatory cells, smooth muscle proliferation and migration. The success of lipophilic S-nitrosothiols will, therefore, depend on the relative contribution of each factor to the restenotic

process. The concentration and site of S-nitrosothiol retention will also affect their ability to attenuate the extent of each factor in the development of restenosis. S-nitrosothiol retention will be determined by a number of factors, such as degree of vessel damage, dosage and means of delivery, all of which offer a means to optimise the angioplasty and drug administration techniques. Also, the release rate of NO from retained S-nitrosothiols will also affect their bioactivity. Appealingly, a slow prolonged release of NO would be more effective in preventing VSMC proliferation, an important factor in neointimal growth after angioplasty (Mooradian *et al.*, 1995). Overall, lipophilic S-nitrosothiols show great promise in the prevention of restenosis following angioplasty, and may also be beneficial in the prevention of thrombosis following coronary artery bypass grafting or used as an adjunct or coating for stents.

Endothelial dysfunction is prevalent in many cardiovascular conditions (see Section 1.7.2). However, in many cases the structural integrity of the endothelium may not necessarily be lost, despite functional impairment (Harrison, 1997; Vallance & Chan, 2001). NO bioavailability may be lost through other mechanisms such as increased oxidant stress (Gryglewski *et al.*, 1986; Nakazono *et al.*, 1991) or desensitisation of vascular smooth muscle (Robinson *et al.*, 1982; Calver *et al.*, 1992), rather than endothelial removal. Therefore, it could be argued that the presence of the intact endothelium would act as a barrier to lipophilic S-nitrosothiols, negating their unique therapeutic potential. However, the loss of endothelium-dependent vasodilatation and the occurrence of fatty streaks are commonly associated and both are early indicators of atherosclerosis (Ross, 1993; Davies, 2000). Therefore, these lipid rich lesions may retain lipophilic S-nitrosothiol, without the complete structural removal of the endothelium. In the later stages of atherosclerosis, endothelial erosion is common and is associated with the formation of microthrombi on the vessel surface (Mann & Young, 1994; Davies, 2000). These areas of erosion present a means for novel S-nitrosothiols to gain access to the subendothelial layers, where their prolonged generation of NO should limit the extent of thrombus formation. By targeting these

regions, other actions of NO come into play, such as the scavenging of oxygen derived radicals and inhibition of lipid peroxidation (Rubbo *et al.*, 1994; Bult *et al.*, 1999; Patel *et al.*, 1999). Therefore, although speculative at present, these novel lipophilic S-nitrosothiols are promising agents in the treatment of vascular disease.

8.6 SUMMARY

This thesis provides insight into the mechanism of action of lipophilic S-nitrosothiols as novel NO donor drugs. The structural adaptations made to form these compounds does not alter their ability to induce sGC-independent vasodilatation, their resistance to tolerance, or their susceptibility to superoxide or thiol depletion. However, the structural adaptations improve the lipophilicity of the compounds, a feature that allows S-nitrosothiols to cause a marked sustained vasodilatation that is selective for areas of endothelial damage. Furthermore, the results of this thesis demonstrate that lipophilic S-nitrosothiols can be used *in vivo* to prevent platelet adhesion to arteries that have been damaged by balloon angioplasty, at a concentration that does not cause significant systemic hypotension. Therefore, novel lipophilic S-nitrosothiols show great therapeutic potential over existing therapies in the treatment of cardiovascular diseases, such as angina, angioplasty and atherosclerosis.

Appendix I

Preparation of solutions

9. APPENDIX I - PREPARATION OF SOLUTIONS

Krebs-Henseleit buffer

Amounts stated are to make 1 L Krebs (final concentration in mM).

	Stock	Normal Krebs		High K^+ Krebs	
NaCl	g/**10% sol.**	6.9 g	(118)	**2.8 ml**	**(4.7)**
$NaHCO_3$	g	1 g	(25)	1 g	(25)
Glucose	g	2.1 g	(5.7)	2.1 g	(5.7)
KCl	10% sol./**g**	3.5 ml	(4.7)	**8.8 g**	**(118)**
$MgSO_4 \cdot 7H_2O$	10% sol.	2.9 ml	(0.6)	2.9 ml	(0.6)
KH_2PO_4	10% sol.	1.6 ml	(1.2)	1.6 ml	(1.2)

Make up to 950 ml. Bubble with 95% O_2, 5% CO_2 for 10 min.

$CaCl_2$	0.68 M	3.7 ml	(2.5)	3.7 ml	(2.5)

Make up to 1 L.

Sodium cacodylate buffer (SCB)

Dissolve 21.4 g sodium cacodylate (0.1 M) in 900ml distilled water.

Adjust to pH 7.4 with HCl (0.1 M).

Make up to 1 L.

TESPA (3-aminopropyltriethoxysilane) slides

Wash slides for 10 s in the following;

1) 10% HCl (S.G.1.18), diluted in 70% alcohol

2) distilled water

3) 100% acetone

Air dry (~1 h).

Ten seconds in following;

1) 2% TEPSA, diluted in 100% acetone

2) 100% acetone

3) 100% acetone

Air dry.

Phosphate Buffered Saline (PBS)

To make 1 litre;

NaCl	80 g
$Na_2HPO_4 2H_2O$	29 g
KH_2PO_4	2 g
KCl	2 g

BSA-PBS

To make 0.5 % BSA;

1 ml BSA (30%) made up to 30 ml with PBS.

Tris-buffered saline (TBS)

To make 1 litre;

Dissolve 6.04 g TRIZMA® base in 800 ml distilled water.

Adjust to pH 7.6 with HCl (S.G.1.18).

Make up to 1 L.

Trypsin-TBS

To make 50 ml;

Dissolve 1 trypsin tablet in 1 ml distilled water.

Add 0.5 ml trypsin solution to 49.5 ml TBS.

Add 3 mg $CaCl_2$.

Appendix II

Permission to reproduce diagrams

PERMISSION TO USE DATA SHOWN IN FIGURE 1.21

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I am writing to you to ask for permission to reproduce two diagrams from the *British Journal of Pharmacology* for use in a Ph.D. thesis. I wish to reproduce the two diagrams showing the vasodilator effects of novel S-nitrosothiols;

Megson, I.L., Grieg, I.R., Gray, G.A., Webb, D.J. & Butler, A.R. (1997). Prolonged effect of a novel S-nitrosated glyco-amino acid in endothelium-denuded rat femoral arteries; potential as a slow release nitric oxide donor drug. *Br. J. Pharmacol.* **122**; p1622; Fig 7.

Megson, I.L., Morton, S., Grieg, I.R., Mazzei, F.A., Field, R.A., Butler, A.R., Caron, G., Gasco, A., Fruttero, R. & Webb, D.J. (1999). N-substituted analogues of S-nitroso-*N*-acetyl-D,L-penicillamine: chemical stability and prolonged nitric oxide mediated vasodilatation in isolated rat femoral arteries. *Br. J. Pharmacol.* **126**; p646; Fig 7.

I wish to use these diagrams in the introduction to my Ph.D. thesis, which focuses on further research using these compounds. The author of these publications, Dr I.L. Megson, is the supervisor of this Ph.D. thesis and has already obtained consent to use these diagrams from the co-authors of these publications. Two copies of this thesis are submitted to the University of Edinburgh library, access to which is granted only to members of the University of Edinburgh library and will not be reproduced elsewhere

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Novel S-nitrosothiols do not engender vascular tolerance and remain effective in glyceryltrinitrate-tolerant rat femoral arteries ☆, ☆☆

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Abstract

Organic nitrates, such as glyceryltrinitrate, are nitric oxide (NO) donor drugs that engender tolerance with long-term use. Here, we tested the hypothesis that our novel S-nitrosothiols, *N*-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-β-D-glucopyranose (RIG200) and S-nitroso-*N*-valeryl-D-penicillamine (D-SNVP), do not induce vascular tolerance ex vivo. Femoral arteries from adult male Wistar rats were preconstricted with phenylephrine and perfused with the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME). Perfusion pressure was measured during 20 h treatment with supramaximal concentrations of NO donor (10 μM). Perfusion with glyceryltrinitrate caused a vasodilatation, which recovered over 2–20 h. In contrast, the S-nitrosothiols caused vasodilatations that were maintained throughout the 20 h perfusion period. Responses to S-nitrosothiols were partially reversed by the NO scavenger ferrohaemoglobin and fully reversed by the soluble guanylate cyclase inhibitor [1H-[1,2,4] oxadiazole [4,3-a]quinoxaline-1-one (ODQ). Glyceryltrinitrate-tolerant vessels were fully responsive to bolus injections of S-nitrosothiols. Resistance to tolerance is an attractive property of our novel compounds, particularly in view of their sustained activity in arteries with damaged endothelium.

Keywords: Nitric oxide (NO); S-Nitrosothiol; Organic nitrate; Tolerance; Blood vessel

1. Introduction

Nitric oxide (NO) is synthesised by endothelial cells in blood vessels (Palmer et al., 1987, 1988; Palmer and Moncada, 1989) and stimulates smooth muscle cell soluble guanylate cyclase, leading to relaxation of vascular tissue (Waldman and Murad, 1987). Synthesis of NO is now recognised to be a major factor in the control of blood pressure and local blood flow in animals (Aisaka et al., 1989; Rees et al., 1989; Gardiner et al., 1990; Chu et al., 1991) and man (Vallance et al., 1989; Haynes et al., 1993). Delivery of exogenous NO to areas of diminished NO activity (Drexler et al., 1991; Calver et al., 1992a,b) is an attractive therapeutic option in the management of many cardiovascular conditions.

Organic nitrates are the most commonly used NO donor drugs in cardiovascular medicine. Glyceryltrinitrate (Fig. 1(a)) is currently used for angina, and for symptomatic relief in severe cardiac ischaemia, myocardial infarction and heart failure. The beneficial action of nitrates is thought to involve NO-mediated systemic venodilatation and dilatation of large arteries, including affected coronary arteries, resulting in reduced venous return and increased blood flow to cardiac tissue (Abrams, 1985). However, the therapeutic use of nitrates is limited by the development of tolerance, where a diminished effectiveness of these drugs is seen after 24 h of continuous therapy (Parker and Fung, 1984). Tolerance can be demonstrated ex vivo, suggesting impairment of a direct vascular mechanism, such as inefficient biotransformation of glyceryltrinitrate (Brien et al., 1986; Slack et al., 1989), or desensitization of the target

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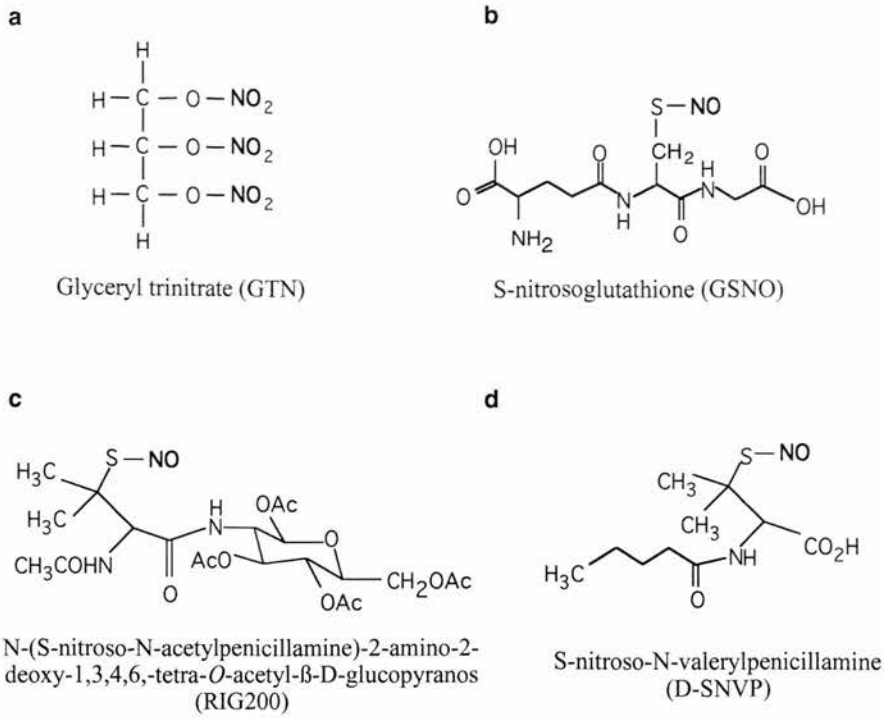

Fig. 1. Structural formulae and full generic names for NO donors used in this study: (a) glyceryltrinitrate (GTN); (b) S-nitrosoglutathione (GSNO); (c) RIG200; (d) D-SNVP.

enzyme, guanylate cyclase (Needleman and Johnson, 1973; Axelsson and Andersson, 1983; Waldman et al., 1986). Recently, in vivo and clinical evidence suggest that continuous nitrate therapy is associated with elevated superoxide production from the endothelium (Munzel et al., 1995, 1996). Superoxide reacts with NO, forming cytotoxic products, such as peroxynitrite, and reducing NO bioavailability (White et al., 1994).

S-Nitrosothiols (general formula R—S—N=O) are nitrosated derivatives of sulphydryl-containing compounds, some of which have been identified as endogenous vasodilators (Stamler et al., 1992a,b). S-Nitrosothiols do not require biotransformation to activate guanylate cyclase, suggesting that S-nitrosothiols may not induce self-tolerance. Indeed, one such agent, S-nitroso-*N*-acetylpenicillamine has been shown to develop less tolerance than glyceryltrinitrate and to remain effective in glyceryltrinitrate-tolerant vessels ex vivo (Kowaluk et al., 1987; Kowaluk and Fung, 1990; Matsumoto et al., 1995) and in vivo (Bauer and Fung, 1991; Shaffer et al., 1992).

Most existing S-nitrosothiols, including S-nitroso-*N*-acetylpenicillamine and S-nitrosoglutathione (Fig. 1(b)), rapidly decompose in an unpredictable manner due to the catalytic effect of trace Cu^+ ions (Dicks et al., 1996; Gordge et al., 1996), thus limiting their therapeutic potential (Megson et al., 1997). We have recently described several novel S-nitrosothiols. *N*-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-ß-D-glucopyranose (RIG200) consists of S-nitroso-*N*-acetylpenicillamine coupled to glucosamine tetra-acetate by an amide bond (Fig. 1(c); Megson et al., 1997) and S-nitroso-*N*-valeryl-D-penicillamine (D-SNVP) which is an *N*-substituted analogue of S-nitroso-*N*-acetylpenicillamine, with a five carbon side-chain (Fig. 1(d); Megson et al., 1999). Both compounds are significantly more stable than S-nitroso-*N*-acetylpenicillamine in solution (half-life; ~40 and ~220 min for S-nitroso-*N*-acetylpenicillamine and RIG200, respectively, D-SNVP exhibits a similar rate of decomposition to RIG200), and are less susceptible to trace Cu^+-catalyzed decomposition (Megson et al., 1997, 1999). Another potential advantage of these compounds over existing NO donors is that they induce sustained vasodilatation in endothelium-denuded rat femoral arteries, suggesting that they may be able to selectively deliver NO to areas of endothelial damage (Megson et al., 1997, 1999).

For these benefits to be maximally exploited therapeutically, it would be valuable if these novel compounds did not engender tolerance with continued use. Here, we used an isolated rat femoral artery model of nitrate tolerance to test the hypothesis that RIG200 and D-SNVP do not induce vascular tolerance or show cross-tolerance with glyceryltrinitrate.

2. Materials and methods

2.1. Preparation

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (300–450 g;

$n = 60$) in a perfusion system described previously (Megson et al., 1997). Briefly, animals were killed by cervical dislocation and both femoral arteries were dissected free. Segments of the artery (7–8 mm long) were cannulated immediately distal to the epigastric arterial branch. The vessels were transferred to Perspex organ chambers (1 ml volume) where they were perfused (0.6 ml min^{-1}: Gilson miniplus 3; Anachem, Luton, UK) and superfused (1 ml min^{-1}: Watson Marlow 302S; Watson Marlow, Falmouth, UK) with fresh oxygenated (95% O_2, 5% CO_2) Krebs buffer solution (composition in mM: NaCl 118, $NaHCO_3$ 25, Glucose 5.7, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 2.5; dissolved in distilled and de-ionised water) at 37°C. The contractile state of the vessel was measured by perfusion pressure, monitored by a differential pressure transducer (T; Sensym SCX 15ANC; Farnell Electronic Components, Leeds, UK). The apparatus permits exclusive drug delivery to the luminal surface of the vessel in the

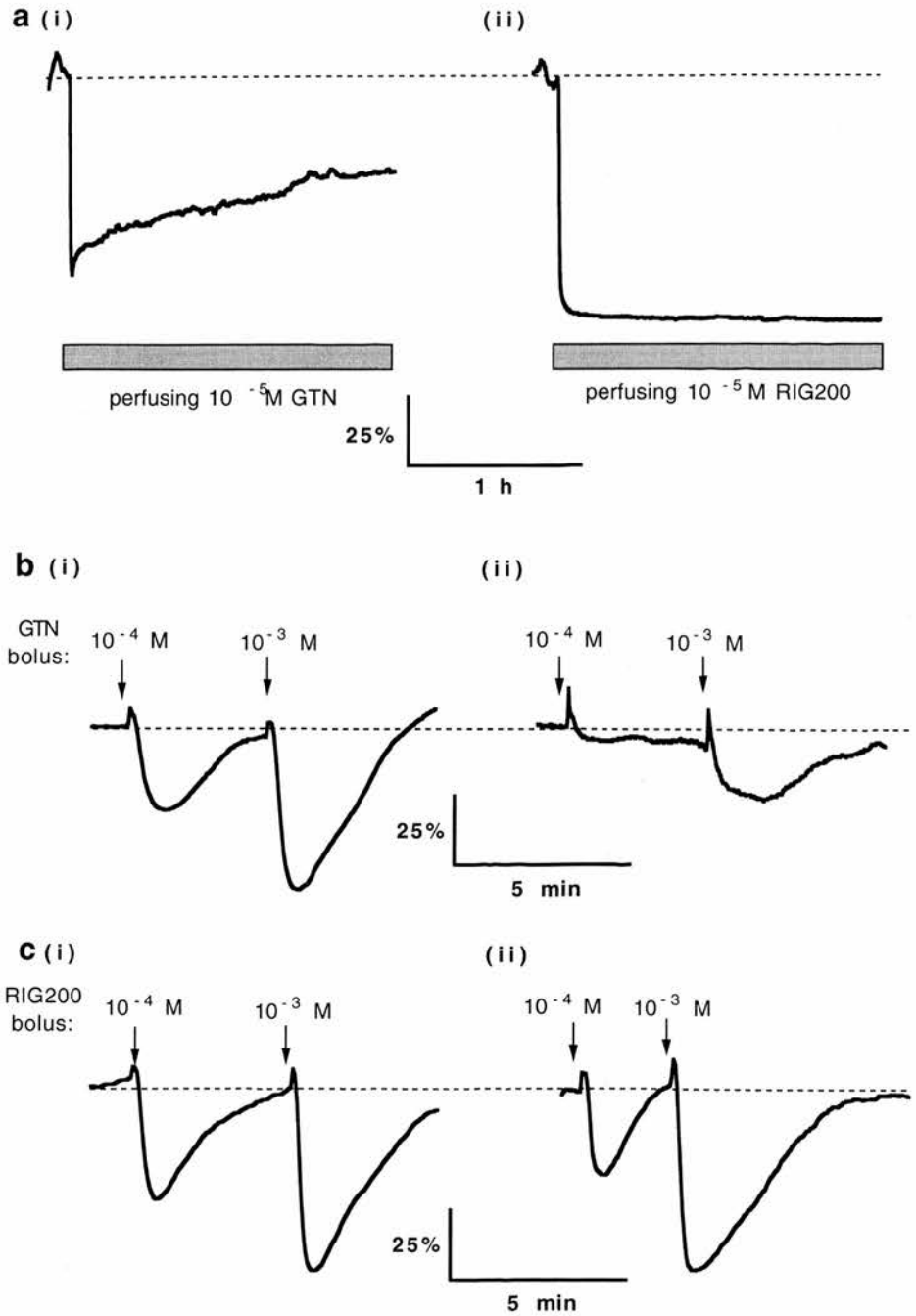

Fig. 2. Pressure recordings showing vasodilator responses. (a) Continuous perfusion (10^{-5} M; 0.6 ml min^{-1}) of (i) GTN or (ii) RIG200. (b) Responses to sequential micro-injections of GTN (10 μl; 10^{-4}, 10^{-3} M) into the perfusate of (i) control and (ii) GTN-tolerant vessels. (c) Responses to sequential micro-injections of RIG200 (10 μl; 10^{-4}, 10^{-3} M) into the perfusate of (i) control and (ii) GTN-tolerant vessels.

internal perfusate or by bolus injection (10 μl) through a resealable rubber septum into the perfusate, immediately upstream of the vessel (transit time to artery ~3 s, through lumen ~300 ms).

All experiments were carried out in a darkened laboratory in order to protect photolabile drugs and to prevent photorelaxation of vessels (Megson et al., 1995).

Vessels were preconstricted with phenylephrine (2–10 μM) in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; Rees et al., 1990) to exclude endothelial and inducible NO-synthase activation in vasodilator responses. Preliminary experiments were carried out to minimise the concentration of L-NAME (20 μM) used to produce a supramaximal response. An L-NAME-induced increase in pressure of >40% of the existing phenylephrine-induced tone was indicative of an active endothelium.

2.2. Experimental protocols

2.2.1. Induction of tolerance

Vessels were perfused with equivalent concentrations of NO donor (10 μM), or Krebs as a control, and perfusion pressure was monitored for 2 h. Phenylephrine was then removed from the internal perfusate and the perfusion rate lowered to 0.1 ml min^{-1} overnight, at 25°C, to optimise vessel survival. At $t = 20$ h, the original phenylephrine-containing solution was re-perfused at the original flow rate (0.6 ml min^{-1}), at 37°C.

2.2.2. Cross-tolerance

In glyceryltrinitrate-treated and control vessels ($t = 20$ h), bolus injections of increasing concentrations of NO donor (10 μl; 10^{-8}–10^{-3} M) were made sequentially into the perfusate. Responses were deemed to have recovered once pressure was maintained for more than 2.5 min, at which time the next concentration was injected.

2.2.3. Washout of NO donor

To confirm viability in vessels that did not re-develop tone with phenylephrine following 20 h of S-nitrosothiol perfusion, the S-nitrosothiol was washed out and the time taken for maximum pressure to be restored was measured.

2.2.4. Nature of NO donor vasodilatation

In S-nitrosothiol-treated vessels at $t = 20$ h, the NO scavenger, ferrohaemoglobin (10 μM; Martin et al., 1985) was added to the internal perfusate, and subsequently, to the superfusate to allow ferrohaemoglobin to infiltrate the vascular smooth muscle, as it has been shown previously that the endothelium may act as a barrier to ferrohaemoglobin (Foley et al., 1993). Responses were deemed complete after pressure was maintained for 5 min. A supramaximal concentration of the soluble guanylate cyclase inhibitor [1H-[1,2,4] oxadiazole [4,3-a]quinoxaline-1-one (ODQ; Garthwaite et al., 1995) was added to the internal perfusate and rapidly washed out once pressure had reached plateau.

2.3. Drugs and reagents

RIG200 was synthesised by a published method (Megson et al., 1997). D-SNVP was synthesised by the following procedure. Sodium valerate was synthesised by reacting sodium hydroxide pellets (8 g, 0.2 mol) in distilled water (100 ml) with valeric acid (21.6 ml, 200 mmol). Sodium valerate (3.9 g, 40 mmol) was added to D-penicillamine (3 g, 20 mmol) in a chilled solution of tetrahydrofuran:water, 4:1 (20 ml). Valeric anhydride (4 ml, 20 mmol) was added and the mixture stirred at room temperature overnight. The tetrahydrofuran was evaporated in vacuo and water (30 ml) was added. Concentrated HCl was added dropwise until a white precipitate formed. This was filtered and recrystallised from hexane to yield *N*-valeryl-D-penicillamine, which was then dissolved in a minimum amount of dichloromethane. Concentrated HCl was dropped onto sodium nitrite and the resulting nitrogen dioxide were bubbled into the penicillamine solution to yield *N*-valeryl-S-nitroso-D-penicillamine. Identity of the products was confirmed by mass spectrometry (EPSRC Mass Spectroscopy Service, University College, Wales, UK) and nuclear magnetic resonance spectroscopy. ODQ was obtained from Tocris Cookson (Langford, Bristol, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK) Methaemoglobin was reduced to the ferro-form with sodium dithionite (fivefold excess; 57.4 μM) as described previously (Martin et al., 1985).

All drugs were stored as solids and dissolved on the day of use with the exception of ferrohaemoglobin, aliquots of which were stored at −70°C and used within 1 month. All

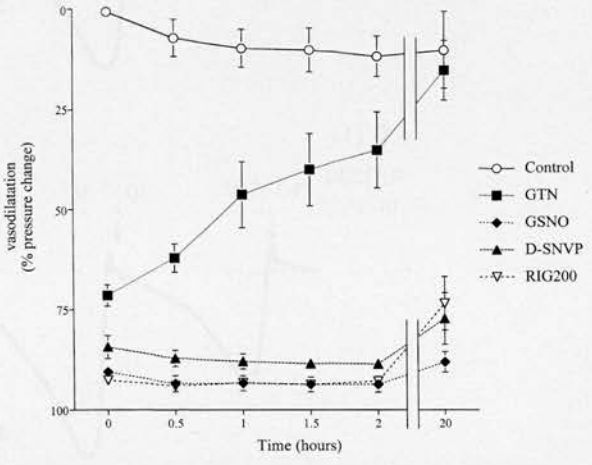

Fig. 3. Effect of perfusing NO donors (10 μM) on perfusion pressure in preconstricted rat femoral arteries. Vasodilatation at 100% represents abolition of tone, with NO donor perfusion beginning at $t = 0$ h. Points shown are means with vertical lines indicating S.E.M. ($n = 45$, 33, 15, 12, 12 for control, GTN, GSNO, RIG200 and D-SNVP respectively).

drugs were diluted in Krebs buffer or saline with the exception of ODQ, which was dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the perfusate was $< 0.1\%$ and preliminary experiments showed that this concentration of DMSO does not affect vessel tone.

2.4. Analysis of results

Signals from pressure transducers were processed by a MacLab/4e analogue-digital converter and displayed through Chart software (AD Instruments, Sussex, UK) on a Macintosh Performa 630 microcomputer.

Vasodilator response amplitude is the decrease in pressure, expressed as a percentage of preconstriction pressure existing before the application of each drug concentration (percent pressure change; positive values represent vasodilatation, where 100% represents maximum possible vasodilatation). Mean values are given $\pm$ standard error of the mean (S.E.M.).

P-values in the text were obtained by two-factor, unrelated analysis of variance (ANOVA). Paired and unpaired, two-tailed Student's *t*-tests were also used where appropriate. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Preconstriction of femoral arteries

Vessels were preconstricted with phenylephrine (6.7 ± 0.3 μM) to give pressures of ~ 50 mm Hg (49 ± 3 mm Hg; $n = 60$). L-NAME (20 μM) led to a $151 \pm 13\%$ increase of pre-existing phenylephrine-induced pressure (110 ± 5 mm Hg; $n = 60$). Bolus injections of drug vehicle (Krebs buffer or saline) had no effect on perfusion pressure.

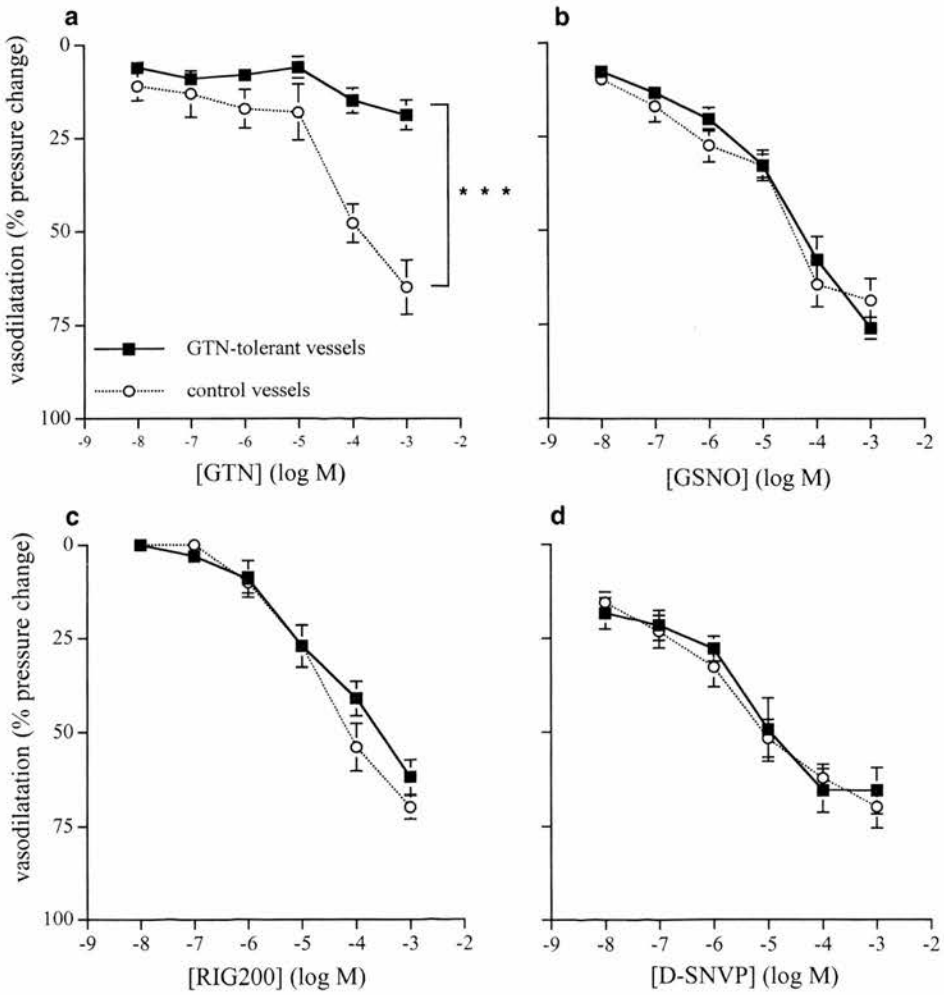

Fig. 4. Log concentration response curves showing the vasodilator effect (% pressure change) of bolus microinjections (10 μl) of (a) GTN, (b) GSNO, (c) RIG200, (d) D-SNVP in control (open circles) and GTN-tolerant (filled squares) vessels. Points shown are means with vertical lines indicating S.E.M. ($n = 6$–9). * * * represents statistical significance at $P < 0.001$ (two-factor, unrelated ANOVA).

3.2. Vasodilator responses to continuous NO donor perfusion

Perfusion of glyceryltrinitrate (10 μM) caused an initial vasodilatation of 72 ± 3% ($n = 33$). Pressure gradually recovered to 35 ± 10% vasodilatation remaining at $t = 2$ h (Figs. 2(a) and 3). After overnight incubation with glyceryltrinitrate ($t = 20$ h), pressure was not significantly different from control (10 ± 10% below preconstriction; $P = 0.64$; 2-tailed, unpaired Student's t-test; $n = 45$).

Perfusion of supramaximal concentrations of S-nitrosothiols (10 μM) produced greater vasodilatation (91 ± 2%, 93 ± 1%, 84 ± 3% for S-nitrosoglutathione, RIG200 and D-SNVP, respectively; $n = 12$–15), which were maintained throughout the 20 h period of perfusion (Figs. 2(a) and 3).

3.3. Vasodilator responses to bolus injections of NO donors in control and glyceryltrinitrate-tolerant vessels

Bolus injections of glyceryltrinitrate (10 μl; 10^{-8}–10^{-3} M) produced transient vasodilatations which recovered within 5 min. In vessels perfused overnight in the absence of glyceryltrinitrate (control), the highest concentration of glyceryltrinitrate tested (10^{-3} M) produced a vasodilatation of 65 ± 7% ($n = 6$). In vessels perfused with glyceryltrinitrate for 20 h (glyceryltrinitrate-tolerant vessels) the response to 10^{-3} M GTN bolus was markedly attenuated (19 ± 4%; $P < 0.001$; two-factor, unrelated ANOVA; $n = 7$; Figs. 2(b) and 4(a)).

Equivalent injections of S-nitrosothiols also produced transient vasodilatations of a similar amplitude to glyceryltrinitrate (69 ± 6%, 70 ± 3% and 70 ± 6% for S-nitrosoglutathione, RIG200 and D-SNVP, respectively; Figs. 2(c) and 4(b), (c), (d)). However, the concentration response curves for the S-nitrosothiols in glyceryltrinitrate-tolerant vessels were not significantly different from those in control vessels ($P > 0.21$; two-factor, unrelated ANOVA; $n = 6$–9).

3.4. Washout of S-nitrosothiols

At $t = 20$ h, the internal perfusate was replaced with Krebs solution containing PE and L-NAME, but without NO donor. On washout of S-nitrosoglutathione, RIG200 or D-SNVP, pressure recovered to levels that were not significantly different from the preconstriction pressure before the perfusion of NO donor ($P = 0.15$; 2-tailed, paired Student's t-test; $n = 18$). Pressure rapidly recovered in 5.5 ± 0.9, 6.5 ± 0.8 and 11.1 ± 4.5 min, respectively ($n = 6$ for all). The time course of D-SNVP washout was significantly slower than S-nitrosoglutathione and RIG200 ($P = 0.001$; two-way, unrelated ANOVA).

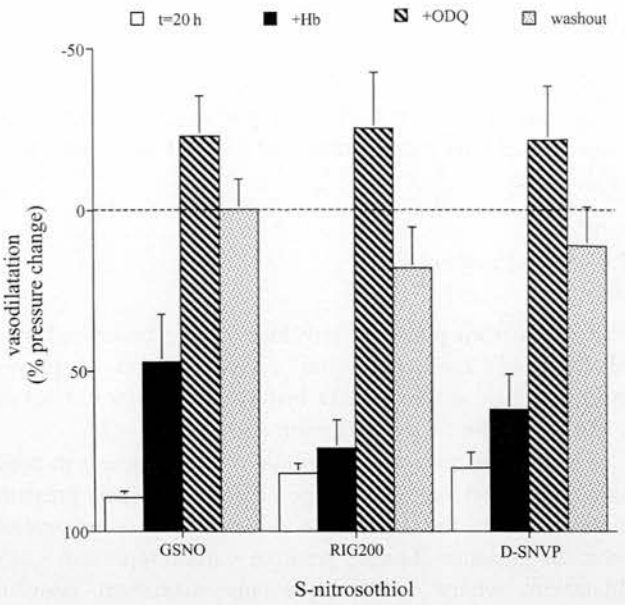

Fig. 5. The effect of ferrohaemoglobin (ferrohaemoglobin; 10 μM), ODQ (20 μM) and S-nitrosothiol washout on the vasodilatation produced by perfusing S-nitrosothiols (10 μM) for 20 h. Points shown are means with vertical lines indicating S.E.M. ($n = 6$ for all). Preconstriction pressure is represented by the dotted line.

3.5. Reversal of S-nitrosothiol vasodilatation with ferrohaemoglobin and ODQ

Following perfusion of S-nitrosoglutathione for 20 h, addition of ferrohaemoglobin (10 μM) to the internal perfusate caused a significant increase in pressure of ~ 40% ($P = 0.005$, 2-tailed, paired Student's t-test; $n = 6$). ferrohaemoglobin had no effect on the vasodilatation produced by RIG200 or D-SNVP ($P > 0.15$ for both; $n = 6$; Fig. 5). Addition of ferrohaemoglobin (10 μM) to the external perfusate caused no additional effect ($n = 6$).

Full restoration of pressure could be achieved by the addition of the soluble guanylate cyclase inhibitor, ODQ (20 μM), to the internal perfusate (Fig. 5). The perfusion pressure in the presence of these compounds was not significantly different from the preconstriction pressure, before addition of NO donor ($P = 0.73$; 2-tailed, paired Student's t-test; $n = 18$).

Treatment of control vessels with ferrohaemoglobin and ODQ had no significant effect on perfusion pressure ($P > 0.05$; paired Student's t-test; $n = 6$).

4. Discussion

Our results show that tolerance to glyceryltrinitrate develops rapidly in rat femoral arteries, within 20 h of continuous exposure, resulting in a marked attenuation of the responses to additional bolus concentrations of glyceryltrinitrate. The endogenous S-nitrosothiol, S-nitrosoglutathione, and novel S-nitrosothiols, RIG200 and D-

SNVP, did not induce tolerance within 20 h or exhibit cross-tolerance in vessels made tolerant to glyceryltrinitrate.

The amplitude of glyceryltrinitrate-induced vasodilatation diminished despite continued perfusion of the drug, and was not evident after 20 h. Down-regulation of NO synthase by NO from glyceryltrinitrate (Moncada et al., 1991) could be excluded as a possible explanation for the results because the NO synthase inhibitor, L-NAME, was present throughout. Given the long duration of the experiments it was necessary to continuously perfuse L-NAME to prevent NO generation from the inducible form, as well as the constitutive, NO synthase. The vasodilator response to boluses of high concentrations of glyceryltrinitrate (10^{-4}, 10^{-3} M) were attenuated in the presence of perfusing glyceryltrinitrate (10^{-5} M), confirming nitrate tolerance in these vessels. Perfused isolated femoral arteries are, therefore, an effective ex vivo model for the investigation of nitrate tolerance and cross-tolerance to NO donor drugs. In addition, tolerance to glyceryltrinitrate can be induced rapidly, facilitating studies investigating the prevention and reversal of nitrate tolerance.

All the S-nitrosothiols (10 μM) that were investigated relaxed arteries to a similar extent, producing ~90% vasodilatation. Vasodilatation to S-nitrosothiols was maintained throughout the 20 h perfusion period despite the slow decomposition of the compounds in the perfusate reservoir. Our results demonstrate that, despite structural modifications, RIG200 and D-SNVP retain the characteristics of existing S-nitrosothiols in that they do not engender tolerance (Kowaluk et al., 1987; Kowaluk and Fung, 1990; Bauer and Fung, 1991). Following 20 h S-nitrosothiol perfusion, pressure was rapidly restored by washing out the S-nitrosothiol, confirming the reversibility of the effect and indicating that the vessel was still viable. RIG200 and D-SNVP have previously been demonstrated to induce a vasodilatation which persists after washout in endothelium-denuded vessels (Megson et al., 1997, 1999). Therefore, the rapid restoration of pressure following S-nitrosothiol washout suggests that the endothelium was functionally intact after 20 h, consistent with the vasoconstrictor effect of L-NAME at the beginning of the experiment. D-SNVP took significantly longer to wash out than S-nitrosoglutathione and RIG200, perhaps reflecting its greater lipophilicity (Megson et al, 1999).

S-Nitrosoglutathione-induced vasodilatation was partially inhibited by perfusion with ferrohaemoglobin, suggesting that extracellular decomposition of S-nitrosoglutathione to release NO contributes to the vasodilatation in response to this compound. This reflects the sensitivity of S-nitrosoglutathione to catalyzed decomposition by metal ions in Krebs solution (Dicks et al., 1996; Gordge et al., 1996), or by elements of the vascular cell surface (Kowaluk and Fung, 1990; Al-Sa'doni et al., 1997). The vasodilatation produced by RIG200 and D-SNVP was unaffected by ferrohaemoglobin perfusion, suggesting that ferrohaemoglobin does not have access to the site where RIG200 and D-SNVP exert their bioactivity. S-Nitrosothiol-induced vasodilatation could be completely reversed by addition of the soluble guanylate cyclase inhibitor, ODQ, suggesting that in this vascular tissue, unlike in platelets (Gordge et al., 1998), the action of S-nitrosothiols is entirely mediated by activation of this enzyme.

Our finding that S-nitrosothiols do not induce tolerance implies that the underlying cause of nitrate tolerance ex vivo is upstream of NO release. Desensitization of the target enzyme, guanylate cyclase (Needleman and Johnson, 1973; Waldman et al., 1986), or upregulation of cGMP-phosphodiesterase activity (Axelsson and Andersson, 1983) have been suggested as potential mechanisms in tolerance development. However, our results show that S-nitrosothiols remain fully active in glyceryltrinitrate-tolerant vessels through a mechanism entirely mediated by guanylate cyclase. Our results also question the involvement of superoxide generation in tolerance development ex vivo, because S-nitrosothiols retained full activity in glyceryltrinitrate-tolerant vessels. S-Nitrosothiols may release NO at a site inaccessible to scavenging by superoxide or possibly stimulate guanylate cyclase directly. However, elevated superoxide levels would at least be able to inactivate extracellular NO from S-nitrosoglutathione in tolerant vessels. The role of superoxide production may, however, be more prominent in vivo, where neurohormonal mechanisms including the renin–angiotensin and endothelin systems may exacerbate oxidative stress (Munzel and Bassenge, 1996).

All the S-nitrosothiols tested were as effective in glyceryltrinitrate-tolerant vessels as in control vessels. This reinforces our conclusion that events prior to NO release or S-nitrosothiol formation (Ignarro et al., 1981) limit the effectiveness of glyceryltrinitrate in tolerance. S-Nitrosothiols decompose spontaneously in solution at varying rates to generate NO (Williams, 1985) and therefore, may not be dependent on the same co-factors needed to release NO from glyceryltrinitrate. In addition, the ability of S-nitrosothiols to directly transfer NO to reduced tissue thiols without the release of free NO (Askew et al., 1995) could lead to activation of guanylate cyclase through nitrosation of cysteine residues in the enzyme (Ignarro et al., 1981). This property could be the underlying reason why S-nitrosothiols do not induce tolerance.

In summary, we have shown that two novel NO donor drugs, RIG200 and D-SNVP do not induce tolerance with 20 h of continuous perfusion, in an ex vivo model of tolerance. In addition, they retain full vasodilator potency in vessels made tolerant to glyceryltrinitrate, despite its continued presence. Our results lend weight to the argument that RIG200 and D-SNVP may be viable clinical alternatives to organic nitrates and existing S-nitrosothiols, because, added to their previously described increased stability and selectivity for areas of endothelial damage, they do not appear to engender tolerance. These features

suggest that RIG200 and D-SNVP could have potential benefits in the treatment of a number of cardiovascular diseases including angina, atherosclerosis, cardiac ischaemia, heart failure, and other conditions where long-term and high dose vasodilator therapy is required.

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